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FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
85286	
U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR)	
10/031308	

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA00/00824	17/07/2000	15/07/1999

TITLE OF INVENTION

Antisense Oligonucleotides for Metabotropic Glutamate Receptor Type 1(MGLUR1)

APPLICANT(S) FOR DO/EO/US

Marian E. Fundytus, Terence J. Coderre, S. Robin Cohen, James L. Henry and Anneli Vainio

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☒ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR 1)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
10/031308	PCT/CA00/00824	85286

24. The following fees are submitted:.		CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :			
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1040.00**		
☒ International prcliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$890.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$740.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$710.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =		**$890.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	19 - 20 =	0	x $18.00	**$0.00**	
Independent claims	2 - 3 =	0	x $84.00	**$0.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$890.00**	
☒ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$445.00**	
SUBTOTAL =				**$445.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$445.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐				**$0.00**	
TOTAL FEES ENCLOSED =				**$445.00**	
				Amount to be: refunded	$
				charged	$

a. ☒ A check in the amount of **$445.00** to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **23-0920** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

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Gerald T. Shekleton
NAME

27,466
REGISTRATION NUMBER

January 14, 2002
DATE

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CERTIFICATE OF MAILING BY "EXPRESS MAIL" (37 CFR 1.10) Applicant(s): **Fundytus et al.**			10/031508 Docket No. 85286
Serial No. **not yet assigned**	Filing Date **herewith**	Examiner	Group Art Unit

Invention:

Antisense Oligonucleotides for Metabotrophic Glutamate Receptor Type 1 (MGLUR1)

I hereby certify that the following correspondence:

National Phase Filing of United States Patent Application and related papers

(Identify type of correspondence)

is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 in an envelope addressed to: The Assistant Commissioner for Patents, Washington, D.C. 20231 on

01/14/2002
(Date)

James Cato
(Typed or Printed Name of Person Mailing Correspondence)

James C. Cato
(Signature of Person Mailing Correspondence)

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Note: Each paper must have its own certificate of mailing.

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Application Data Sheet

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Application Information

Title Line One:	Antisense Oligonucleotides For Metabotropic Glutamate Receptor Type 1 (MGLUR1)
Total Drawing Sheets:	23
Formal Drawings:	Yes
Application Type:	Utility
Docket Number:	85286

Representative Information

Registration Number One:	24,003
Registration Number Two:	22,839
Registration Number Three:	28,903
Registration Number Four:	27,429
Registration Number Five:	25,060
Registration Number Six:	22,053
Registration Number Seven:	27,466
Registration Number Eight:	29,434
Registration Number Nine:	29,054
Registration Number Ten:	29,381
Registration Number Eleven:	34,044
Registration Number Twelve:	27,600
Registration Number Thirteen:	34,137
Registration Number Fourteen:	39,724
Registration Number Fifteen:	37,963
Registration Number Sixteen:	41,050
Registration Number Seventeen:	34,217

FOREIGN PRIORITY INFORMATION

This application is based on International Application

Application One:	PCT/CA00/00824
Filing Date:	17/07/2000

Application Data Sheet

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Filing Date:	17/07/2000
Priority Date:	15/07/1999

10/031308
531 Rec'd PCT/PT 15 JAN 2002

85286 PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In Re U.S. Patent Application	)
	)
Applicant: Fundytus et al.	)
	)
Serial No.: Not Yet Assigned	)
	)
Filed: Herewith	)
	)
For: Antisense Oligonucleotides For Metabotropic Glutamate Receptor Type 1 (MGLUR1)	)

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

This is a Preliminary Amendment for entry in the above-identified application.

In the Claims:

Please amend the claims as follows:

7. (amended) A composition comprising the antisense oligonucleotide of claim 1.

12. (amended) A method according to claim 9, wherein said mGluR1 is human mGluR1a.

13. (amended) An oligonucleotide according to claim 1, wherein said mGluR1 is from a species excluding rat.

15. (amended) The antisense oligonucleotide of claim 1 comprising a nucleotide sequence having form 13 to 22 bases in length, and hybridizing to a portion of said mRNA 3 bases prior to the initiation codon of said gene and continuing to

the stop condon of said gene.

16. (amended) The use of antisense oligonucleotide according to claim 1, to treat chronic pain.

IN THE ABSTRACT

Please add the enclosed page to serve as the Abstract.

Respectfully submitted,

WELSH & KATZ, LTD.

By
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Dated: ~~January 14, 2002~~

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10/031308
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VERSION WITH MARKINGS TO SHOW CHANGES MADE

7. (amended) A composition comprising the antisense oligonucleotide of [any one of claims 1 to 6] ~~claim 1~~.

12. (amended) A method according to [any one of claims 9 to 11] ~~claim 9~~, wherein said $mGluR_1$ is human $mGluR_{1a}$.

13. (amended) An oligonucleotide according to [any one of claims 1 to 6] ~~claim 1~~, wherein said $mGluR_1$ is from a species excluding rat.

15. (amended) The antisense oligonucleotide of [any one of claims 1 to 6] ~~claim 1~~ comprising a nucleotide sequence having form 13 to 22 bases in length, and hybridizing to a portion of said mRNA 3 bases prior to the initiation codon of said gene and continuing to the stop condon of said gene.

16. (amended) The use of antisense oligonucleotide according to [any one of claims 1 to 6] ~~claim 1~~, to treat chronic pain.

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10/031308
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OLIGONUCLEOTIDES FOR METABOTROPIC GLUTAMATE RECEPTOR TYPE 1 ($MGLUR_1$)

FIELD OF THE INVENTION

The invention relates to antisense oligonucleotides for metabotropic glutamate receptor type 1 ($mGluR_1$).

BACKGROUND OF THE INVENTION

Metabolic glutamate receptor type 1 ($mGluR_1$), is a member of group I mGluRs and is positively coupled to phosphatidylinositol (PI) hydrolysis. Activation of this type of receptor ultimately leads to activation of protein kinase C (PKC) and increased concentrations of intracellular Ca^{2+}, thereby causing initiation of cellular excitation. Furthermore, activity at group I mGluRs (including $mGluR_1$) has been shown to enhance activity at N-methyl-D-aspartate (NMDA) receptors via a PKC mediated mechanism (Raymond *et al* (1994) *J Physiol Paris* **88**:181-192; Bleakman *et al* (1992) *Mol Pharmacol* **42**:192-196; Chen and Huang (1992) *Nature* **356**:521-523.; Harvey and Collingridge (1993) *Br J Pharmacol* **109**:1085-1090; Kelso *et al* (1992) *J Physiol* **449**:705-718; and Kitamura *et al* (1993) *J Neurochem* **61**: 100-109). It has recently been shown that, in rats, treatment with an antisense oligonucleotide targeting $mGluR_1$ reduces NMDA receptor activity (Fundytus *et al* (1999) *Society for Neuroscience Abstracts* **25**: 449). Thus, antisense oligonucleotides targeting $mGluR_1$ have potential therapeutic benefit in any injury or disease state in which glutamate and NMDA receptor activity have been shown to be involved. These include chronic pain (neuropathic, inflammatory), neurodegenerative disorders, central nervous system (CNS; brain and spinal cord) trauma, stroke and ischemia, and gastrointestinal disorders, as well as tumor suppression.

Stroke and ischemia

Glutamate neurotoxicity has been shown to be involved in several models of ischemia. For example, in *in vitro* models mimicking conditions of hypoxia/ischemia and glucose deprivation

in brain and spinal cord slices, it has been shown that glutamate release is increased, and this effect is attenuated by glutamate receptor antagonists (Nakai *et al* (1999) *Eur J Pharmacol* 366(2-3):143-150; Kimura *et al* (1998) *J Pharmacol Exp Ther* 285(1):178-185; Reyes *et al* (1998) *Brain Res* 782(1-2): 212-218). Neuronal damage in these models is attenuated by agents that inhibit glutamate release (Culmsee *et* al (1998) *Eur J Pharmacol* 342(2-3): 193-201).

There are also several *in vivo* animal models of ischemia. In models of focal cerebral ischemia, induced by occlusion of one or more arteries and resembling stroke in humans, it has been shown that glutamate release is enhanced and that glutamate receptor antagonists attenuate neuronal damage (Caragine *et al* (1998) *Brain Res* **793**: 255-264; O'Neill *et al* (1998) *Neuropharmacology* **37**: 1211-1222; Minger *et al* (1998) *Brain Res* **810**: 1881-1899; Dexter *et al* (1998) *Anesthesia & Analgesia* **87**: 72-78). These phenomena have also been shown in animal models of global ischemia, resembling that seen in humans following a heart attack or during thoracoabdominal surgery (Lang-Lazdunski *et al* (1999) *J Thoracic & Cardiovascular Surgery* **117**: 881-889). Inhibition of glutamate release is also neuroprotective (Crumrine *et al* (1997) *Stroke* **28**: 2230-2236; Culmsee *et al* (1998) *Eur J Pharmacol* **342**: 193-201; Haseldonckx *et al* (1997) *Stroke* **28**: 428-432). It has been suggested that active modulation of mGluR activity in cerebral ischemia may prevent neuronal injury (Maiese (1998) *Clinical Neuropharmacology* **21**: 1-7). PKC has also been implicated in both focal and global ischemic neuronal injury, and inhibition of PKC has been shown to attenuate measures associated with ischemic injury (Nakane *et al* (1998) *Brain Res* **782**: 290-296; Sieber *et al* (1998) *Stroke* 29(7): 1445-1452). It has also been suggested that Ca^{2+} is one of the triggers involved in ischemic cell death (Kristian and Siesjo (1998) Stroke **29**: 705-718; Stys (1998) *J Cerebral Blood Flow & Metabolism* **18**: 2-25). Other investigators have suggested that glutamate can induce astroglial cell swelling by an interaction with mGluRs, and that this plays an important role in the early development of brain injuries caused by ischemia (stroke or brain trauma) (Hansson *et al* (1997) *Acta Neurochirurgica* - Suppl. **70**: 148-151). To verify the involvement of mGluRs in ischemic neuronal damage, it has been shown that the mGluR agonist, (1S,3R)-ACPD significantly enhances hippocampal damage due to global ischemia (Henrich-Noack and Reymann (1999)Eur J Pharmacol **365**; 55-58). Heat stroke induced ischemia is also

accompanied by an increase in glutamate release, and the symptoms are attenuated by pre-treatment with NMDA antagonists in rats (Yang *et al* (1998) *Brain Res* **795**(1-2): 121-127).

Glutamate has also been implicated in ocular ischemia associated with glaucoma, retinal ischemia due to central artery occlusion, anterior ischemic optic neuropathy, and possibly also optic neuritis, optic nerve trauma and AIDS (Sucher *et al* (1997)*Vision Res* **37**: 3483-3493; Lagreze *et al* (1998) *Investigative Ophthalmology & Visual Science* **39**: 1063-1066; Duarte *et al* (1998*) Gen Pharmacol* **30**: 289-295). Actions at mGluRs may play an important role (Duarte *et al* (1998) *supra*). Moreover, inhibition of PKC (associated with activity at group I mGluRs) has been shown to significantly attenuate damage in a rat model of ocular ischemia (Hicks *et al* (1998) *Gen Pharmacol* **30**: 265-273).

The role of glutamate in ischemia and stroke has also been examined in the clinic. There are increased CSF levels of glutamate in encephalitis (Launes *et al* (1998) *NeuroReport* **9**: 577-581) and in blood and CSF of acute stroke patients (Castillo *et al* (1997) *Lancet* **349**: 70-83). Increased levels of glutamate and aspartate in CSF drawn by lumbar puncture have been associated with neurological deficits in patients undergoing thoracoabdominal aortic aneurysm surgery (Brock *et al* (1997) *Annals of Thoracic Surgery* **64**: 999-1003). Antagonists at NMDA receptors have been shown to ameliorate the extent of the infarction with cerebral ischemia (Lees (1997) *Neurology* **49**(5 suppl 4): S66-S69; Dyker and Lees (1999) *Stroke* **30**: 986-992).

Central nervous system trauma

The mechanisms of neuronal injury in central nervous system (CNS; brain and spinal cord) appear to be very similar to those observed in ischemia. Thus, glutamate excitotoxicity plays an important role in CNS trauma, as may be sustained by a blow to the head, spinal cord injury, whiplash, noise-induced hearing loss, etc. In an *in vitro* model of post-traumatic neuronal death, $mGluR_1$ antagonists afforded significant neuroprotection either alone, or in combination with NMDA receptor antagonists (Mukhin *et al* (1997) *NeuroReport* **8**: 2561-2566), whereas a group I mGluR agonist exacerbated neuronal injury. The effect of activation of group I mGluRs was only partially alleviated by an NMDA antagonist, suggesting that

mGluR effects are mediated only partially via NMDA receptor modulation, and mGluR antagonists may have therapeutic potential on their own (Mukhin *et al* (1997) *supra*).

Animal models show that glutamate release is enhanced following trauma (Malda *et al* (1998) *J Neurotrauma* **15**: 655-664). Treatments that inhibit glutamate release have been shown to be neuroprotective in rat models of CNS trauma (Zhang *et al* (1998) *J Neurosci Res* **52**: 342-349). Treatment with NMDA antagonists is neuroprotective in animal models of CNS trauma (Phillips *et al* (1998) *Hippocampus* **8**: 390-401; Puel *et al* (1998) *NeuroReport* **9**: 2109-2114).

Glutamate neurotoxicity has also been associated with CNS trauma in the clinic. In severely head injured patients, levels of excitatory amino acids (EAAs) are elevated, secondary ischemic brain injury and focal contusions are most strongly associated with high levels of EAAs, and sustained high intracranial pressure and poor outcome are significantly correlated with high levels of EAAs (Bullock *et al* (1998) *J Neurosurgery* **89**: 507-518).

Neurodegenerative disorders

Neurodegenerative disorders such as Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis (ALS), Huntington's disease, mitochrondrial encephalomyopaythies, spinocerebellar degeneration syndromes, motor neuron diseases, and even schizophrenia have been linked to glutamate excitotoxicity (Massieu and Garcia (1998) *Neurobiology (Budapest)* **6**: 99-108; Arias *et al* (1998) *Neurobiology (Budapest)* **6**: 33-43; Bittigau and Ikonomidou (1997) *J Child Neurology* **12**: 471-485; Myhrer (1998) *Neurosci & Biobehav Rev* **2391**: 131-139). Glutamate neurotoxicity leads to production of beta amyloid, associated with Alzheimer's disease (Meier-Ruge and Bertoni-Freddari (1997) *Annals NY Acad Sci* **826**: 229-241. Intracerebral administration of kainic acid to rats produces limbic-cortical neuronal damage that has been compared to the neuropathology of schizophrenia (Csernansky *et al* (1998) *Biol Psychiatry* **44**: 1143-1150). In an animal model of Parkinsonism, it has been shown that NMDA is involved in degeneration of dopamine neurons of the substantia nigra (Sonsalla *et al* (1998) *Amino Acids* **14**: 69-74; Loopuijt and Schmidt (1998) *Amino Acids* **14**: 17-23 . It has also been suggested that AIDS dementia may be associated with glutamate induced neuronal injury (Lipton (1998) *Ann Rev Pharmacol Toxicol* **38**: 159-177). Morover,

glutamate excitotoxicity has been implicated in seizure activity and epilepsy, with the contribution of NMDA, AMPA/kainate and metabotropic glutamate receptors (Loscher (1998) *Prog Neurobiol* **54**: 721-741).

Gastrointestinal disorders

Some evidence has accumulated that glutamate neurotoxicity may be involved in gastrointestinal disorders. Recently, it has been suggested that glutamate receptors on the spinal cord dorsal horn may be involved in functional gastrointestinal disorders (Mayer et al (1999) *Canadian J of Gastroenterology* **13** Suppl A: 65A-70A). Other investigators have shown that prolonged stimulation of enteric ganglia by glutamate caused necrosis and apoptosis of enteric neurons (Kirchgessner *et al* (1997) *J Neurosci* **17**: 8804-8816), effects mimicked by NMDA, and blocked by the NMDA antagonist AP5. These authors suggest that overactivation of enteric glutamate receptors may contribute to intestinal damage produced by anoxia, ischemia and excitotoxins present in food (Kirchgessner *et al* (1997) *supra*).

Tumor suppression

An added benefit of antisense oligonucleotide knockdown of $mGluR_1$ may be suppression of tumor growth in cancer patients. As discussed earlier, $mGluR_1$ is positively coupled to PI hydrolysis, and activation of these receptors leads to the activation of protein kinase C (PKC). Knockdown of $mGluR_1$ has been shown to inhibit the activation of PKC (Fundytus *et al* (1999) *Society for Neuroscience Abstracts* **25**: 449). Inhibition of PKC, with antisense oligonucleotides targeting PKC, has been shown to inhibit tumor growth (Dean *et al* (1996) *Cancer Res* **56**: 3499-3507).

Opioid tolerance and dependence

A major problem associated with the treatment of advanced cancer is the control of pain. This difficulty is exacerbated by the fact that different types of pain such as somatic, visceral, incident and neuropathic are not all equally amenable to current analgesic therapies. Often, neuropathic pain is only partially relieved by the most common analgesics, opioids (Cherny et al (1994) *Neurology* **44**: 857-861; MacDonald (1991) *Recent Results in Cancer Res* **121**: 24-35; McQuay et al (1992) *Anesthesia* **47**: 757-767). Opioid therapy is problematic for 30% of cancer patients who experience pain, and of these, 2/3 have neuropathic pain.

Currently, the most common analgesics are opioid drugs. However, administration of opioids is often associated with undesirable side effects such as sedation, respiratory depression, nausea, vomiting, constipation, pruritis, renal toxicity.

Since the 1970's, many investigators have shown that glutamate is involved in the development of opioid tolerance and dependence. Glutamate release is enhanced during opioid withdrawal (Jhamandas *et al* (1996) *J Neurosci* **16**: 2758-2766). Most investigators have concentrated on the NMDA glutamate receptor, and they have shown that treatment with NMDA antagonists attenuates the development of tolerance, and alleviates the severity of opioid withdrawal symptoms (Koyuncuoglu *et al* (1990) *Pharmacol Biochem & Behav* **35**: 829-832; Trujillo and Akil (1991) Science **251**: 85-87; Trujillo and Akil (1994) Brain Res **633**: 178-188; Mao *et al* (1995) *Pain* **62**: 259-274; Marek *et al* (1991) *Brain Res* **547**: 77-81; Marek *et al* (1991) *Brain Res* **558**: 163-165). More recently, it has been shown that treatment with antagonists at group I mGluRs, as well as chronic inhibition of products of PI hydrolysis (PKC activation and intracellular Ca^{2+} release) significantly reduces the severity of opioid withdrawal (Fundytus and Coderre (1994) Br J Pharmacol **113**: 1215-1220; Fundytus and Coderre (1996) *Eur J Pharmacol* 300: 173-181; Fundytus and Coderre (1999) *Pain Forum* **8**: 3-13; Fundytus and Coderre (1999) *Pain Forum* **8**: 58-63. Very recent results from our laboratory show that treatment with an antisense oligonucleotide targeting $mGluR_1$ in rats attenuates the development of morphine tolerance (Sharif *et al* (2000) in preparation). We have also demonstrated that antisense oligonucleotide knockdown of $mGluR_1$ reduces PKC activity (Fundytus *et al* (2000) *Br. J. Pharmacol.* submitted).

There is some evidence that glutamate receptor antagonists may also be useful to treat opioid addiction in the clinic. Koyuncuoglu and Saydam (1990) *J Clinical Pharmacol, Therapy, & Toxicol* **28**: 147-152, showed that the NMDA antagonist dextromethorphan reduces symptoms of withdrawal in heroin addicts.

Antisense Oligonucleotides

Current technology allows for direct targeting of genes with antisense oligonucleotides, which are purported to reduce protein synthesis. Oligonucleotides complementary to, and designed to hybridize to, a target mRNA transcript via Watson-Crick base pairing are used. Formation of an oligonucleotide-mRNA duplex leads to mRNA inactivation and inhibition of protein synthesis.

There are currently several clinical trials evaluating the efficacy of oligonucleotides given systemically to humans to combat the AIDS virus and cancer tumor growth (Akhtar and Agrawal (1997) *Trends Pharmacol Sci* **18**: 12-18. These trials employ phosphorothioate-modified oligonucleotides. There is toxicity to the liver, kidney and spleen associated with systemically administered phosphorothioate-bonded oligonucleotides, as well as necrosis of central nervous system tissues with local administration.

The present invention provides antisense oligonucleotides directed toward human $mGluR_1$ which are useful in the treatment of diseases and disorders involving glutamate and/or $mGluR_1$ activity imbalance, including but not limited to stroke, ischemia, pain, etc.

This background information is provided for the purpose of making known information believed by the applicant to be of possible relevance to the present invention. No admission is necessarily intended, nor should be construed, that any of the preceding information constitutes prior art against the present invention.

SUMMARY OF THE INVENTION

An object of the present invention is to provide antisense oligonucleotides for metabotropic glutamate receptor type 1 ($mGluR_1$). In accordance with an aspect of the present invention, there is provided antisense oligonucleotides of human metabotropic glutamate receptor type I.

In accordance with another aspect of the present invention there is provided a use of an antisense oligonucleotide of human metabotropic glutamate receptor type I in the treatment of a disease or disorder characterized by an imbalance of glutamate and/or $mGluR_1$ activity.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: Western blot analysis. Sample Western blots and histogram results from Western blot analysis of lumbar spinal cords and the thalamus/periaqueductal region of brains taken from ACSF-, AS- and MS-treated rats after 7 days of oligonucleotide infusion, and 4 days after nerve constriction (induced by implantation of a cuff around one sciatic nerve) or sham-surgery (n = 3 per group).

A Change in spinal binding density of anti-rat $mGluR_1$ IgG, compared to ACSF-treated rats, in AS- and MS-treated rats. AS treatment induced a 43.85% decrease of $mGluR_1$ protein compared to vehicle treatment in neuropathic rats, and a 39.22% decrease in $mGluR_1$ protein compared to vehicle treatment in sham-operated rats. The amount of $mGluR_1$ protein in lumbar spinal cord from MS-treated rats was not decreased (and even appeared to be increased) from vehicle-treated rats.

B Change in binding density of anti-rat $mGluR_1$ IgG in thalamus/periaqueductal region of brain of AS- and MS-treated rats, compared to ACSF-treated rats. AS treatment induced a 24.62% decrease in neuropathic rats, and a 17.80% decrease in $mGluR_1$ protein in sham-operated rats.

C Change in binding density of anti-rat mGluR5 IgG in lumbar spinal cord of AS- and MS-treated rats, compared to ACSF-treated rats. AS treatment induced a slight decrease in $mGluR_5$ protein in neuropathic rats (7.67%), and a more pronounced decrease in sham-operated rats (25.63%).

Figure 2: DHPG-induced spontaneous nociceptive behaviours. Mean time spent exhibiting nociceptive behaviour after i.t. injection of 50 nmol DHPG in ACSF- (n = 8), AS- (n = 5) and MS-treated (n =5) rats. (* significantly different from ACSF-treated ($p < 0.05$); † significantly different from MS-treated ($p < 0.05$))

Figure 3: Nerve-injury induced hyperalgesia and allodynia. Change in response to cold, heat and mechanical stimulation on days 4, 8, 12 and 16 days (pre-treatment group) or 4, 8, 12 and 18 days (post-treatment group) after nerve constriction in neuropathic and sham-operated rats treated intrathecally with either ACSF (cuffed, n = 12 pre-treatment, n = 6 post-treatment; sham, n = 4 pre-treatment), AS (cuffed, n = 9 pre-treatment, n = 5 post-treatment; sham, n = 3 pre-treatment) or MS (cuffed, n = 7 pre-treatment, n = 6 post-treatment; sham, n = 4 pre-treatment).

A *Cold water test in pre-treatment group:* Mean increase in number of responses (lifting of hindpaw) when rats stood in water at 1°C. There was a significant interaction between neuropathic condition and i.t. treatment ($F_{(2,33)} = 7.91$, $p < 0.01$). Post-hoc tests showed that AS-treated neuropathic rats had a significantly lower response frequency across test days compared to ACSF- or MS-treated neuropathic rats.

B *Cold water test in post-treatment group:* Mean increase in number of responses after nerve injury, both prior to i.t. infusion (day 4), and after i.t. infusion (days 8, 12 and 18). All nerve injured rats showed a large increase in response frequency in the ipsilateral hindpaw, compared to baseline, on day 4 after nerve injury, prior to i.t. oligonucleotide infusion. The dotted line at day 5 indicates when the i.t. infusion of oligonucleotides began. There was a significant drug x day interaction ($F_{(6,42)} = 2.30$, $p = 0.05$). Post-hoc Fisher's LSD t-tests showed that after i.t. infusion, AS-treated neuropathic rats showed a significantly lower increase in response frequency on days 8, 12 and 18 after nerve injury, compared to ACSF- and MS-treated rats regardless of test day.

C *von Frey hair test in pre-treatment group:* Mean percent decrease in 50% response threshold in grams. There were significant effects of neuropathic condition ($F_{(1,33)} = 98.44$, $p < 0.01$), i.t. treatment ($F(2,33) = 9.23$, $p < 0.01$), and test day ($F(3,99) = 3.48$, $p < 0.05$). Post-hoc tests showed that neuropathic AS-treated rats had a significantly lower decrease in 50% response threshold than either ACSF- or MS-treated rats.

D *von Frey hair test in post-treatment group:* Mean percent decrease in 50% response threshold in grams. On day 4 after nerve injury, prior to i.t. drug infusion, all neuropathic rats showed a large decrease in 50% response threshold. The dotted line at day 5 indicates when the i.t. infusion of oligonucleotides began. There was a significant drug x day interaction ($F_{(6,42)} = 3.35$, $p < 0.05$), and post-hoc tests showed that after i.t. infusion,

neuropathic AS-treated rats had a significantly lower decrease in 50% response threshold than either ACSF- or MS-treated rats

E *Radiant heat plantar test in pre-treatment group:* Mean percent decrease in response latency. There were significant effects of interaction between neuropathic condition and i.t. treatment ($F_{(2,33)} = 3.26$, $p = 0.05$), and test day ($F_{(3,99)} = 6.57$, $p < 0.01$). Post-hoc tests Fisher's LSD t-tests showed that AS-treated neuropathic rats had a significantly lower decrease in response latency than ACSF- or MS-treated neuropathic rats.

F *Radiant heat plantar test in post-treatment group:* Mean percent decrease in response latency. All nerve injured rats showed a large decrease in response latency, compared to baseline, on day 4 after nerve injury, prior to i.t. drug infusion. The dotted line at day 5 indicates when the i.t. infusion of oligonucleotides began. There was a significant drug x day interaction ($F_{(6.42)} = 6.22$, $p < 0.05$), and post-hoc tests showed that after i.t. infusion, AS-treated neuropathic rats had a significantly lower decrease in response latency from days 8 to 18 after nerve injury.

Figure 4: Morphine dose-response curve. Morphine dose-response curve in neuropathic and sham-operated rats 4 days after nerve injury for ACSF- (n = 15 each for cuffed and sham-operated), AS- (n = 16, cuffed) and MS-treated (n = 17, cuffed) rats. ACSF- and MS-treated neuropathic rats showed little analgesia after morphine was injected intrathecally (ED_{50} = 63.71 μg (21.38 μg-1950 mg) and 705.28 μg (95% C.I. not calculable) respectively). In contrast, AS-treated neuropathic rats showed a robust analgesic effect, and were not different from sham-operated rats (ED_{50} = 8.13 μg (5.25-12.02 μg) and 9.72 μg (4.68-20.89 μg) respectively).

Figure 5: [3H]PDBu binding. [^{3}H]PDBu binding autoradiography in lumbar spinal cord of neuropathic and sham-operated rats.

A Histogram summary showing the density of [^{3}H]PDBu binding (nCi g^{-1}) in ACSF-, AS- and MS-treated neuropathic and sham-operated rats (n = 3 rats per treatment combination, with 5 slides from each rat, for a total of 15 slides per group). Planned comparisons showed that ACSF-treated sham-operated rats had a lower binding density than ACSF-treated neuropathic rats; AS-treated neuropathic rats had a lower binding density than

either ACSF- or MS-treated neuropathic rats. (* significantly different from ACSF-treated neuropathic rats; # significantly different from MS-treated neuropathic rats)

B Computer-generated image (MCID) of a single representative slide from each group showing sample binding of [^{3}H]PDBu in ACSF-, AS- and MS-treated neuropathic and sham-operated rats.

Figure 6: NMDA-induced spontaneous nociceptive behaviours. Mean time spent exhibiting nociceptive behavior in neuropathic (n = 4-6 per dose) and sham-operated (n = 4-6 per dose) rats given increasing doses of i.t. NMDA; and in neuropathic rats treated with either AS (n = 11) or MS (n =10), and given 2.5 nmol of NMDA. (*significantly different from ACSF-treated ($p < 0.05$); † significantly different from MS-treated ($p < 0.05$); # significantly different from sham-operated ($p < 0.05$))

Figure 7: Mechanical allodynia in neuropathic rats treated with i.t. AS, MS or ACSF as indicated by mean percent decrease in 50% response threshold (grams) to von Frey hair stimulation of the plantar surface of the hindpaw. There was a significant day by i.t. treatment interaction ($F_{(6,42)} = 3.35$, $p< 0.05$). All rats displayed mechanical allodynia four days after nerve injury, indicated by a large decrease in 50% response threshold from baseline. Drug infusion began on day 5 after nerve injury (indicated by the dotted line), and on days 8 to 18 after nerve injury, post-hoc tests showed that AS treated rats displayed a significantly attenuated reduction in 50% response threshold compared to ACSF and MS treated rats.

Figure 8: Heat hyperalgesia in neuropathic rats treated with i.t. AS, MS or ACSF as indicated by mean percent decrease in response latency to focussed radiant heat applied to the plantar surface of the hindpaw. There was a significant i.t. treatment by day interaction ($F_{(6.42)} = 6.22$, $p< 0.05$). All rats displayed heat hyperalgesia four days after nerve injury, indicated by a large decrease in response latency from baseline. Drug infusion began on day 5 (indicated by the dotted line), and on days 8 to 18 after nerve injury, post-hoc tests showed AS treated rats exhibited a significantly attenuated reduction in response latency, compared to ACSF and MS treated rats.

Figure 9: Cold hyperalgesia in neuropathic rats treated with i.t. AS, MS or ACSF as indicated by increase in number of responses (hindpaw lifts) when rats stood in a 1 cm deep 1°C water bath for 75 sec. There was a significant i.t. treatment by day interaction ($F_{(6,42)} = 2.30$, $p = 0.05$). All rats displayed cold hyperalgesia four days after nerve injury, indicated by a large increase in response frequency. Drug infusion began on day 5 (indicated by the dotted line), and on days 8 to 18 after nerve injury, post-hoc tests indicated that AS treated rats exhibited a significantly attenuated increase in response frequency, compared to ACSF and MS treated rats.

Figure 10: Inflammation (CFA)-induced hyperalgesia and allodynia. Change in response to heat (A, B) and mechanical (C, D) stimulation of the CFA injected and contralateral hindpaws on days 1 to 8 after CFA injection.

A Radiant heat plantar test in pre-treated group: Mean percent decrease in response latency in ACSF-, AS- and MS-treated rats. ANOVA indicated a significant i.t. treatment by foot interaction ($F_{(2, 32)} = 5.93$, $p < 0.01$), a significant i.t. treatment by day interaction ($F_{8, 128)} = 2.52$, $p < 0.05$), and a significant foot by day interaction ($F_{(4, 128)} = 8.29$, $p < 0.01$). Post-hoc Fisher's LSD t-tests indicated that the percent decrease in withdrawal latency of the injected paw was significantly attenuated in AS-treated rats compared to ACSF- and MS-treated rats, while there was no difference between groups in the contralateral hindpaw. Post-hoc Fisher's LSD t-tests showed that AS-treatment attenuated heat hyperalgesia on days 1, 4, 6 and 8, but not on day 2 following CFA injection.

B Radiant heat plantar test in post-treated group: Mean percent decrease in response latency in ACSF-, AS- and MS-treated rats. Oligonucleotide infusion began on day 2 after CFA injection (indicated by the dotted line). ANOVA indicated a significant i.t. treatment by foot by day interaction ($F_{(6, 90)} = 2.93$, $p < 0.05$). Post-hoc Fisher's LSD t-tests showed that the decrease in withdrawal latency was significantly greater in the injected paw for all i.t. treatment groups on day 1 following CFA injection, prior to oligonucleotide infusion. On days 4 to 8 after CFA injection, the decrease in withdrawal latency of the injected paw was significantly attenuated in AS-treated rats compared to ACSF- and MS-treated rats, and in AS-treated rats the paw withdrawal latency was not different between injected and contralateral paws.

C Von-Frey test in pre-treated group: Mean percent decrease in 50% response threshold in ACSF-, AS- and MS-treated rats. ANOVA indicated a significant i.t. treatment by foot interaction ($F_{(2, 30)} = 4.63$, $p < 0.05$), and a significant foot by day interaction ($F_{(3, 90)} = 3.42$, $p < 0.05$). Post-hoc Fisher's LSD t-tests indicated that the decrease in 50% response threshold in the injected hindpaw was attenuated in AS-treated rats, compared to ACSF- and MS-treated rats.

D Von-Frey test in post-treated group: Mean percent decrease in 50% response threshold in ACSF-, AS- and MS-treated rats. Oligonucleotide infusion began on day 2 after CFA injection (indicated by dotted line). ANOVA indicated a significant i.t. treatment by foot interaction ($F_{(2, 32)} = 8.03$, $p < 0.01$). Post-hoc Fisher's LSD t-tests showed that the decrease in 50% response threshold in the injected paw was attenuated in AS-treated rats, compared to ACSF- and MS-treated rats (from days 4 to 8 after CFA injection).

Figure 11: Western Blot Analysis. Sample Western blots and histogram summary results from Western blot analysis of lumbar spinal cords taken from naïve rats (no treatment), and ACSF-, AS- and MS-treated CFA-injected rats after 7 days of oligonucleotide infusion.

A Peak binding density of $mGluR_1$ IgG in lumbar spinal cords taken from naïve, and ACSF-, AS- and MS-treated CFA-injected rats.

B Change in binding density, compared to either naïve or ACSF-treated CFA-injected, of $mGluR_1$ IgG in lumbar spinal cords of AS- and MS-treated CFA-injected rats. In ACSF- and MS-treated, CFA-injected rats, there was a slight increase (+20.55%) in $mGluR_1$ protein in lumbar spinal cord compared to naïve rats. There was no difference in $mGluR_1$ protein between ACSF- and MS- treated rats (MS-treated versus ACSF-treated = -3.93%). In contrast, the amount of $mGluR_1$ protein was greatly decreased in lumbar spinal cords of AS-treated, CFA-injected rats compared to either naïve rats (-52.46%) or ACSF-treated, CFA-injected rats (-60.56%).

C Sample Western blot showing binding of $mGluR_1$ IgG to the $mGluR_1$ protein.

Figure 12: Growth curve (indicated by mean cell count; n = 3) for SH-SY5Y cells incubated for 48, 72, 96 or 168 hours with either vehicle (medium) alone, or 10 μM antisense (AS) Sequence ID #1, or the missense (MS) control. ANOVA indicated that there are no

significant differences between treatment groups at any time point. Note that this sequence was manufactured by GMP methods.

Figure 13: Duplicated experiment showing the growth curve (indicated by mean cell count; n = 3) for SH-SY5Y cells incubated for 48, 72, 96 or 168 hours with either vehicle (medium) alone, or 10 μM antisense (AS) Sequence ID #1, or the missense (MS) control. ANOVA indicated that there are no significant differences between treatment groups. Note that this sequence was manufactured by GMP methods.

Figure 14: Mean percent mortality (n = 3) for SH-SY5Y cells incubated for 48, 72, 96 or 168 hours with either vehicle (medium) alone, or 10 μM antisense (AS) Sequence ID #1, or the missense (MS) control. ANOVA indicated a significant treatment by day interaction ($F_{(6, 18)} = 2.59$, $p = 0.05$) . Post-hoc Fisher's LSD t-tests indicated that vehicle-treated cells showed significantly more mortality at 48 hours compared to all other time points. Fisher's LSD t-tests also showed that vehicle-treated cells displayed higher mortality at 48 hours than either AS- or MS-treated cells.

Figure 15: Duplicated experiment showing mean percent mortality (n =3) for SH-SY5Y cells incubated for 48, 72, 96 or 168 hours with either vehicle (medium) alone, or 10 μM antisense (AS) Sequence ID #1, or missense (MS) control. ANOVA indicated a significant treatment by day interaction ($F_{(6, 18)} = 6.04$, $p < 0.01$). Post-hoc Fisher's LSD t-tests indicated that vehicle-treated cells displayed significantly higher mortality at 72 and 168 hours than at either 48 or 96 hours. Fisher's LSD t-tests also indicated that vehicle-treated cells displayed significantly higher mortality at 72 and 168 hours than AS-treated cells, and significantly higher mortality at 72 hours than MS-treated cells. Fisher's LSD t-tests also indicated that AS-treated cells displayed significantly higher mortality at 48 hours than vehicle-treated cells, but significantly less mortality at all other time points.

Figure 16: Growth curve (indicated by mean cell count; n = 3) for SH-SY5Y cells incubated for 48, 72, 96 or 168 hours with either vehicle (medium) alone, or 10 μM antisense (AS)

Sequence ID #3, or the missense (MS) control. ANOVA indicated that there are no significant differences between treatment groups at any time point.

Figure 17: Growth curve (indicated by mean cell count; n = 3) for SH-SY5Y cells incubated for 48, 72, 96 or 168 hours with either vehicle (medium) alone, or 10 μM antisense (AS) Sequence ID #5, or the missense (MS) control. ANOVA indicated that there are no significant differences between treatment groups at any time point.

Figure 18: Growth curve (indicated by mean cell count; n = 3) for SH-SY5Y cells incubated for 48, 72, 96 or 168 hours with either vehicle (medium) alone, or 10 μM antisense (AS) Sequence ID #7, or the missense (MS) control. ANOVA indicated that there are no significant differences between treatment groups at any time point.

Figure 19: Western blot analysis and sample Western blot for Sequence ID #3. This antisense (AS) treatment induced a decrease in $mGluR_1$ protein production in comparison to the missense (MS) control treatment, as indicated by area under the curve for $mGluR_1$ IgG binding density.

Figure 20: Western blot analysis and sample Western blot for Sequence ID #5. This antisense (AS) treatment induced a decrease in $mGluR_1$ protein production in comparison to the missense (MS) control treatment, as indicated by area under the curve for $mGluR_1$ IgG binding density.

Figure 21: Western blot analysis and sample Western blot for Sequence ID #7. This antisense (AS) treatment induced a decrease in $mGluR_1$ protein production in comparison to the missense (MS) control treatment, as indicated by area under the curve for $mGluR_1$ IgG binding density.

Figure 22: Western blot analysis and sample Western blot for Sequence ID #1. Antisense (AS) treatment induced a 56.93% decrease in amount of $mGluR_1$ protein as indicated by area under the curve for $mGluR_1$ IgG binding density.

Figure 23: Western blot analysis and sample Western blot for Sequence ID #1. Antisense (AS) treatment induced a 72.21% decrease in amount of $mGluR_1$ protein as indicated by area under the curve for $mGluR_1$ IgG binding density.

Table 1: Groups for animal infusion

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used herein and the laboratory procedures in spectroscopy, drug discovery, cell culture, molecular genetics, plastic manufacture, polymer chemistry, diagnostics, amino acid and nucleic acid chemistry, and sugar chemistry described below are those well known and commonly employed in the art. Standard techniques are typically used for preparation of plastics, signal detection, recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection).

The techniques and procedures are generally performed according to conventional methods in the art and various general references (see generally, Sambrook et al. Molecular Cloning: A Laboratory Manual, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., and Lakowicz, J. R. Principles of Fluorescence Spectroscopy, New York: Plenum Press (1983) for fluorescence techniques, which are incorporated herein by reference) which are provided throughout this document. Standard techniques are used for chemical syntheses, chemical analyses, and biological assays. As employed throughout the disclosure, the following terms, unless otherwise indicated, shall be understood to have the following meanings:

"Corresponds to" refers to a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or

that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

"Naturally-occurring", as used herein, as applied to an object, refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

"Nucleic acid" refers to DNA and RNA and can be either double stranded or single stranded. The invention also includes nucleic acid sequences which are complementary to the claimed nucleic acid sequences.

"Oligonucleotide", as used herein, refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

"Polynucleotide" refers to a polymeric form of nucleotides of at least 10 bases in length, either ribonucleotides or deoxynucleotides or a modified form of either type of nucleotide. The term includes single and double stranded forms of DNA or RNA.

"Protein", as used herein, refers to a whole protein, or fragment thereof, such as a protein domain or a binding site for a second messenger, co-factor, ion, etc. It can be a peptide or an amino acid sequence that functions as a signal for another protein in the system, such as a proteolytic cleavage site.

Other biochemistry and chemistry terms herein are used according to conventional usage in the art, as exemplified by The McGraw-Hill Dictionary of Chemical Terms (ed. Parker, S., 1985), McGraw-Hill, San Francisco).

In one embodiment of the present invention antisense oligonucleotides are designed that are complementary to specific regions of the rat $mGluR_1$ gene. In a preferred embodiment of the present invention antisense oligonucleotides are designed that are complementary to specific regions of the human $mGluR_1$ gene, wherein the $mGluR_1$ gene can be $mGluR_{1\alpha}$ gene. Although the human and rat genes are purported to share significant homology, the antisense sequences used in rats do not target the human gene.

Exemplary antisense oligonucleotide sequences of the present invention are listed below. It should be apparent to one skilled in the art that other antisense oligonucleotide sequences that are complementary to specific regions of the human $mGluR_1$ gene are within the scope of the present invention.

Sequences targeting human mGluR1α, sequence GB-PR1: HSU31215
Coding region bases 236-3820 (gene is 4074 bp) (location indicated in parentheses)

SEQ ID#1: (233-250)	5'-AAG GAG CCC GAC CAT GGT-3'
SEQ ID#2: (236-250)	5'-AAG GAG CCC GAC CAT-3'
SEQ ID#3: (236-253)	5'-CAA AAG GAG CCC GAC CAT-3'
SEQ ID#4: (239-256)	5'-AAA CAA AAG GAG CCC GAC-3'
SEQ ID#5: (242-259)	5'-AAA AAA CAA AAG GAG CCC-3'
SEQ ID#6: (245-262)	5'-GAA AAA AAA CAA AAG GAG-3'
SEQ ID#7: (248-265)	5'-TGG GAA AAA AAA CAA AAG-3'
SEQ ID#8: (251-264)	5'-CGC TGG GAA AAA AAA CAA-3'
SEQ ID#9: (254-267)	5'-GAT CGC TGG GAA AAA AAA-3'
SEQ ID#10: (257-270)	5'-AAA GAT CGC TGG GAA AAA-3'
SEQ ID#11: (260-277)	5'-CAA AAA GAT CGC TGG GAA-3'
SEQ ID#12: (3803-3820)	5'-TTA CAG GGT GGA AGA GCT-3'
SEQ ID#13: (3799-3817)	5'-CAG GGT GGA AGA CGT TTG-3'

SEQ ID#14: (3796-3814) 5'-GGT GGA AGA GCT TTG CTT-3'

Sequences targeting human mGluR1α, GB-PR2: HUMMGLUA
Coding region bases 1-3585 (gene is 6384 bp) (location indicated in parentheses)

SEQ ID#15: (1-18) 5'-CAA AAG GAG CCC GAC CAT-3'
SEQ ID#16: (7-24) 5'-AAA AAA CAA AAG GAG CCC-3'
SEQ ID#17: (13-30) 5'- TGG GAA AAA AAA CAA AAG-3'
SEQ ID#18: (19-36) 5'-GAT CGC TGG GAA AAA AAA-3'
SEQ ID#19: (25-42) 5'-CAA AAA GAT CGC TGG GAA-3'
SEQ ID#20: (3583-3600) 5'-GTG GAC CCT TCC CCC TTA-3'

Sequences targeting human mGluR1β GB-PR1: HSU31216
Coding region bases 1-2721 (gene is 3670 bp) (location indicated in parentheses)

SEQ ID #21: (1-18) 5'-CAA AAG GAG CCC GAC CAT-3'
SEQ ID#22: (7-24) 5'-AAA AAA CAA AAG GAG CCC-3'
SEQ ID#23: (13-30) 5'-TGG GAA AAA AAA CAA AAG-3'
SEQ ID#24: (19-36) 5'-GAT CGC TGG GAA AAA AAA-3'
SEQ ID#25: (25-42) 5'-CAA AAA GAT CGC TGG GAA-3'
SEQ ID#26: (31-48) 5'-CAC CTC CAA AAA GAT CGC-3'
SEQ ID#27: (37-54) 5'-AAG GGA CAC CTC CAA AAA-3'
SEQ ID#28: (2719-2735) 5'-CAG TGT GGG GGT TTT CAA-3'
SEQ ID#29: (2713-2729) 5'-GGG GGT TTT CAA AGC TGC-3'
SEQ ID#30: (2707-2723) 5'-TTT CAA AGC TGC GCA TGT-3'

Sequences targeting human mGluR1β GB-PR2:HUMMGLUB
Coding region bases 371-3091 (gene is 3295 bp long) (location indicated in parentheses)

SEQ ID#31: (365-382) 5'-GAG CCC GAC CAT GGT GGT-3'
SEQ ID#32: (371-388) 5'-CAA AAG GAG CCC GAC CAT-3'
SEQ ID#33: (377-394) 5'-AAA AAA CAA AAG GAG CCC-3'
SEQ ID#34: (383-400) 5'-TGG GAA AAA AAA CAA AAG-3'
SEQ ID#35: (389-406) 5'-GAT CGC TGG GAA AAA AAA-3'
SEQ ID#36: (395-411) 5'-CAA AAA GAT CGC TGG GAA-3'
SEQ ID#37: (3278-3295) 5'-GAA AAG GTC AGG CTC TTG-3'

SEQ ID#38: (3272-3289) 5'-GTC AGG CTC TTG CCA GAG-3'

SEQ ID#39: (3266-3283) 5'-CTC TTG CCA GAG CCT TGG-3'

Preparation of $mGluR_1$ Antisense Oligonucleotides

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to the $mGluR_1$ mRNA or gene, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding $mGluR_1$. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used in vivo to initiate translation of an mRNA molecule transcribed from a gene encoding $mGluR_1$, regardless of the sequence(s) of such codons.

The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon. The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an

mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding nucleotides on the gene. The 5' cap of an mRNA comprises an N^7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the

DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and in the case of *in vitro* assays, under conditions in which the assays are performed.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotides have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes for treatment of cells, tissues and animals, especially humans.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 30 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 nucleobases (i.e. from about 8 to about 30 linked nucleosides). As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure, however, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

In one embodiment of the present invention the antisense oligonucleotides comprise modified oligonucleotide backbones which may or may not include phosphorus atoms.

In other oligonucleotide mimetics of the present invention, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Teaching of PNA compounds can be found in Nielsen *et al.*, Science, 1991, **254**, 1497-1500.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Other preferred modifications include 2'-methoxy (2'-O--CH_3), 2'-aminopropoxy (2'-OCH_2 CH_2 CH_2 NH_2) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5- hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. I., ed. John Wiley & Sons, 1990, those disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, **30**, 613, and those disclosed by Sanghvi, Y. S., Chapter 15, Antisense Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B., ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular

distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al* (1989) *Proc Natl Acad Sci USA* **86**: 6553-6556), cholic acid (Manoharan *et al* (1994) *Bioorg Med Chem Lett* **4:** 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan *et al* (1992) *Ann NY Acad Sci* **660:** 306-309; Manoharan *et al* (1993) *Bioorg Med Chem Lett* **3:** 2765-2770), a thiocholesterol (Oberhauser *et al.*, Nucl. Acids Res., 1992, **20**, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, EMBO J., 1991, **10**, 1111-1118; Kabanov *et al.*, FEBS Lett., 1990, **259**, 327-330; Svinarchuk et al., Biochimie, 1993, 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan *et al.*, Tetrahedron Lett., 1995, **36**, 3651-3654; Shea *et al.*, Nucl. Acids Res., 1990, **18**, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, Nucleosides & Nucleotides, 1995, **14**, 969-973), or adamantane acetic acid (Manoharan *et al.*, Tetrahedron Lett., 1995, **36**, 3651-3654), a palmityl moiety (Mishra *et al.*, Biochim. Biophys. Acta, 1995, **1264**, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al.*, J. Pharmacol. Exp. Ther., 1996, **277**, 923-937.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Consequently, comparable results can often be

obtained with shorter oligonucleotides when chimeric oligonucleotides are used, compared to phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of

endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl)phosphate] derivatives according to the methods disclosed in WO 93/24510 to Gosselin et al., published Dec. 9, 1993 or in WO 94/26764 to Imbach *et al.*

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of $mGluR_1$ is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to reduce pain, to minimize glutamate neurotoxicity and/or excitotoxicity associated with stroke, ischemia, CNS trauma, neurodegenrative disorders, gastrointestinal disorders or to inhibit tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding $mGluR_1$, enabling sandwich hybridization and other assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding $mGluR_1$ can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of $mGluR_1$ in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the

present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Toxicity Studies

It is necessary to confirm that the phosphodiester-bonded oligonucleotides, according to the present invention, are not toxic, even at high doses and that they do not promote tumor growth.

I. Animal models

It has previously been shown that i.t. antisense infusion of antisense targeting $mGluR_1$ in rats reduces neuropathic pain, as well as amount of $mGluR_1$ receptor protein in spinal cord (Fundytus *et al* (1997) *Soc Neurosci Abst* **23**, 1013). It has also been confirmed that the phosphodiester-bonded oligonucleotide used in rats are not toxic, even at high doses. It is generally observed that increasing doses of antisense oligonucleotides do not further reduce protein production once you have reached 30%-50% reduction (Phillips and Gyurko (1995) *Regul Peptides* **59**, 131-141). Because a 39% reduction in protein production has been observed, without toxicity, it is unlikely that higher doses will be toxic. Initial studies used a dose of 50 μg/day, with no observable adverse effects. In contrast to phosphorothioate modified oligonucleotides, phosphodiester-bonded oligonucleotides in concentrations up to 15 mM were not toxic when infused into the intracerebral space of rats (Whitesell *et al*, (1993) Proc. Natl. Acad. Sci. USA **90**, 4665-4669). Phosphodiester oligonucleotides do appear to reach their cellular target, as they are internalized within cells (Stein and Cheng, 1993), and are stable in CSF (Wahlstedt, 1994).

Vehicle- or phosphodiester-bonded antisense and missense oligonucleotides, targeting either rat or human $mGluR_1$, are infused intrathecally (i.t.; at the L4 to L5 lumbar region) in rats, *via* a catheter attached to an osmotic mini-pump, for 14 days (the maximum time of infusion allowed by current osmotic mini-pump technology, which is longer than the 3-day length of treatment employed in human trials), in doses of, for example, 5, 50, 500 and 1000 μg/day. Antisense- and missense-treated animals are compared to vehicle-treated controls, as well as to rats not given infusion. Table 1 outlines the setup of one example of such an experiment using18 experimental groups which corresponds to a total of 108 rats.

(i) Behavioral monitoring

Rats' motor coordination is assessed using the rotarod and righting reflex tests, which are well known to workers skilled in the art. In the rotarod test, rats are placed on a rotating rod (1 revolution per second) and their ability to stay on this rod is assessed as a function of how long they remain on the rod (up to a cutoff time of 60 sec). In the righting reflex test, rats are placed on their backs, and the latency to return to a standing position is measured. Motor function in antisense- and missense-treated rats are compared to motor function in both vehicle-infused and non-infused control animals.

Rats' sensitivity to heat, cold and mechanical stimulation are assessed with the plantar test, cold water test and von Frey hair test respectively, according to procedures known in the art. In the plantar test, the plantar surface of the rat's hindpaw is stimulated with a point of radiant heat, and the latency for paw withdrawal is measured. In the cold water test, the rat is standing in 1 cm deep, 1°C water for 75 sec, and latency to lift a hindpaw, latency to jump, frequency of hindpaw lifts and cumulative duration of hindpaw lifts is recorded. In the von Frey hair test, the plantar surface of the rat's hindpaw is stimulated with thin filaments, using an up-down method (Chaplan *et al*, (1994) J. Neurosci. Methods **53**, 55-63), and a 50% response threshold in grams is calculated. Sensitivity to these stimuli in oligonucleotide-infused rats is compared to that of vehicle infused and non-infused rats.

Rats are weighed daily to determine if oligonucleotide infusion induces weight loss, which may indicate distress. Weight changes are compared between both vehicle-infused and non-infused controls.

(ii) Collection of biological samples

Rats are housed individually in plastic metabolic cages for 1 hour prior to collection of blood and CSF samples. Urine samples are collected from these cages at 1, 6 and 12 hours, then every 24 hours after initiation of drug infusion in separate groups of rats. Collection continues until 4 days after termination of drug infusion.

Blood, approximately 1ml, is collected from a venous catheter implanted in the vena cava (Yashpal, Gauthier and Henry, 1985) using a heparinized 1 ml syringe at 1, 6 and 12 hour, then every 24 hours after beginning drug infusion, until 4 days after termination of drug infusion. To replenish blood volume, an equal volume of physiological saline is injected immediately following collection. The blood plasma is collected according to standard methods known in the art and frozen at -70°C until analyzed.

A second, shorter, intrathecal catheter (extending to the thoracic region) is attached to the catheter used to infuse oligunucleotides, and implanted at the same time (Jhamandas, Yaksh, Harty, Szolcsanyi and Go, 1984). CSF is collected via this second catheter (10-12 µl) at the same time points as blood. CSF samples are immediately frozen at -70°C until analysis.

At the end of the experiment, rats are euthanized and spinal cords and brains collected as described below. If pharmacokinetic analysis indicates that oligonucleotides reach systemic circulation (i.e. they are present in blood and/or urine) we will also collect heart, lungs, liver, spleen and kidneys.

(iii) Measuring oligonucleotides in CSF, plasma and urine samples

The amount of oligonucleotide in biological samples can be determined using high performance liquid chromatography (HPLC) with ultraviolet (UV) detection. The HPLC system may be a Hewlett Packard Series 1050, consisting of a gradient pump, autoinjector and

variable-wavelength UV detector. In one example the anion-exchange chromatography is performed using a 20 × 1 mm I.D. guard column (Upchurch Scientific, Oak Harbor, WA), handpacked with spherical 13 mm Dionex Nucleopak PA-100 support (Dionex Chromatography, Sunnyvale, CA). A ternary solvent gradient elution method can be employed, consisting of three mobile phases: A (25 mM Tris, 1 mM EDTA, pH = 7.0), B (25 mM Tris, 1 mM EDTA, 2M LiBr, pH = 7.0) and C (formamide). In this case the mobile phase composition will initially be set at 60% A, 10% B, 30% C, at a flow rate of 1 ml/min, then be brought to 30% A, 40% B, 30% C over 1.2 min, and held for 0.8 min. Oligonucleotides are detected spectrophotometrically at a wavelength of 267 nm.

CSF, plasma and urine samples can be diluted using 100 µl of 0.5% NP-40 (Sigma, St. Louis, MO) in 0.9% saline. Samples are then centrifuged at 12 000 × g for 10 min, and 50 to 100 µl of supernatant will be injected directly onto the HPLC system

Standard curves for antisense and missense oligonucleotides in CSF, plasma and urine, in concentrations from 0.05 to 8 µM, are prepared daily. These calibration curves are linear, with less than 16% coefficient of variation. Concentrations as low as 0.01 µM were detected using this method (Qian *et al*, (1997) J. Pharmacol. Exp. Ther. **282**, 663-670).

(iv) Pharmacokinetic analysis

Mean oligonucleotide concentration is calculated for each sample, and then for each group at each time point. The area under the curve is obtained using Lagrange polynomial integration from time zero to the last measured sample time, with extrapolation to infinity using the least-squares terminal slope method with the NCOMP computer program (Qian *et al*, (1997) J. Pharmacol. Exp. Ther. **282**, 663-670). From the areas and terminal slopes, clearance (CL), steady state volume of distribution (V_{ss}), half-life ($t_{1/2}$) and maximum concentration (C_{max}) are determined. Data is analyzed with one, two and three compartments and the best fit will be adopted. These data are indicative of the stability of the oligonucleotides in CSF, blood and urine.

(v) Morphological changes in spinal cord and brain

At the end of the experiment, rats are given a lethal dose of sodium pentobarbital, and perfused transcardially with phosphate buffered saline (PBS; pH = 7.4), followed by 4% paraformaldehyde (PFA) in PBS. The spinal cord and brain are removed and placed in 4% PFA overnight. Spinal cord and brain are embedded in paraffin and serial 10 μm cross sections will be cut and stained with hematoxylin and eosin. Morphological changes are analyzed using an image analysis system (Liu *et al*, (1997) J. Neurosci. **17**, 5395-5406). Loss of Nissl staining indicates cell damage. Staining in spinal cords and brains from vehicle-treated rats is compared to staining in spinal cords and brains from non-infused (normal) controls. Staining in spinal cords and brains from oligonucleotide-treated rats will be compared to that of both vehicle-infused and non-infused controls.

(vi) Pathological changes in heart, lung, liver and kidney

Because phosphodiester-bonded oligonucleotides are rapidly degraded in blood, it is unlikely that the oligonucleotides will reach systemic circulation (Akhtar and Agrawal, 1997). However, if pharmacokinetic analysis indicates that the oligonucleotides reach systemic circulation (i.e. oligonucleotide is present in blood and/or urine samples), the heart, lung, liver, spleen, and kidneys are also collected as per their specifications. These tissues are examined for inflammation, edema, hemorrhage, degeneration, necrosis, changes in mitochondria (e.g. swelling), using standard techniques known to those skilled in the art.

II. Human Cell Culture Models

Before using the antisense oligonucleotides of the present invention in human patients, it is necessary to first verify that they are not toxic to human cells, and that they do not promote tumor growth. It is also useful to verify that the antisense targeting human $mGluR_1$ gene will arrest production of receptor protein in human cells, especially cells of the human central nervous system. It should be readily understood by a worker skilled in the art that any human cell line which expresses metabotropic glutamate receptors, for example, human glial and neuroblastoma cells (Lee *et al* (1995) *Proc Natl Acad Sci USA* **92**: 8083-8087), can be used in validation studies.

(i) Toxicity in human cell culture

It is unlikely that the phosphodiester bonded antisense oligonucleotides of the present invention will be toxic (Whitesell *et al* (1993) *Proc Natl Acad Sci USA* **90**: 4665-4669) in human cells. This was confirmed as described in example IV and shown in figures 12 – 18. Standard methods known in the art can be used to determine toxicity in human cell culture. The basic concept is to measure cell growth and/or mortality rates in the presence and in the absence of the test antisense oligonucleotide. Unacceptable toxicity is indicated by greater than 20% more cell death in antisense oligonucleotide treated cells relative to control cells.

(ii) Tumorigenicity in human cell culture

Antisense oligonucleotides directed against $mGluR_1$ are unlikely to promote tumor growth; by inhibiting $mGluR_1$ expression, the activation of PKC will be inhibited (Schoepp and Conn (1993) *Trends Pharmacol Sci* **14**: 13-25), and inhibition of PKC (with oligonucleotides) has been shown to inhibit tumor growth (Dean *et al* (1996) *Cancer Res* **56**: 3499-3507). Standard techniques known in the art can be used in order to confirm that the antisense oligonucleotides of the present invention do not cause tumorgenicity in human cells. Generally, the antisense oligonulceotides are monitored for their ability to affect the growth of a malignant human cell line and compared to negative control cells. A specific example of a tumorgenicity validation test is provided below.

The effects of antisense and missense oligonucleotides on the ability of a malignant human neuroblastoma cell line, for example SK-N-AS, obtained from ATCC, to grow in soft agar can be monitored in order to test for tumorigenicity in central nervous system cells. Cell cultures are grown as described above in growth media containing from 0.2 to 20 mM antisense or missense oligonucleotides, or artificial CSF, and seeded at 5×10^5 cells per 60 mm dish (Simcha *et al* (1996) *J Cell Biol* **133**: 199-209). The number of viable cells can be counted using the trypan blue exclusion assay as described herein at various times after treatment. The rate of growth of oligonucleotide treated cells is compared to untreated and artificial CSF treated cells (an increased rate of growth indicates tumorigenic effects). Because there is a small possibility that the oligonucleotides of the present invention can reach systemic circulation, this test can also be performed in breast (MB-157; ATCC) and lung (DMS-114;

ATCC) cancer cell lines. Breast and lung cancer cells are a good choice because these two types of cancer also commonly metastasize to the central nervous system. Unacceptable tumorigenicity is indicated by >20% excess growth of oligonucleotide treated cells relative to controls.

A test that can be used to further test for tumorgenicity is a thymidine incorporation test using these cell lines to assess rate of DNA synthesis. The cells are incubated with [^{3}H]thymidine (2 μCi/well) for 24 hours, beginning at the 24th hour of culture. Cells are harvested and radioactivity is counted (cpm/well), for example, using a matrix 9600 gas counter (Packard Instrument Company, Meriden, CT). Data from oligonucleotide-treated cells can be compared to control values. Radioactivity counts >20% greater than controls will constitute unacceptable tumorigenicity.

(iii) Efficacy in Cell Culture

Once it is determined that the test antisense oligonucleotide does not cause toxicity or tumorgenicity it is useful to confirm its efficacy *in vivo* prior to its use in clinical trials. The techniques used to study efficacy *in vivo* are standard techniques well known in the art. Generally, to monitor the effect of the test oligonucleotide *in vivo*, human cells expressing metabotropic glutamate receptors are treated with the test oligonucleotide and the amount of $mGluR_1$ produced is compared to that produce in control cells, which are prepferrably cells treated with a missense oligonucleotide.

In vivo efficacy studies using antisense oligonucleotides of the present invention are presented in example V. The data of figures 19 – 23 demonstrate that antisense treatment reduces the amount of $mGluR_1$ produced by human cells in comparison to that produced by the same cells treated with the missense control oligonucleotide.

Clinical Trials in Advanced Cancer Patients (Therapeutic Exploratory Trials)

It would be obvious to one skilled in the art that the antisense oligonucleotides of the present invention would be tested in Clinical Trials in order to obtain regulatory approval for

therapeutic use. An example of standard procedures carried out in a clinical trial is presented below.

Recruitment of patients

To recruit patients, the palliative care physician can identify patients for whom opioid therapy is problematic, and who are scheduled to receive intrathecal (i.t.) drug adminstration for pain control. These patients can be approached in order to explain the goals of the study and obtain written consent from those patients who wish to participate in the study. An expert palliative care physician can then assess the patient. For example, only those patients diagnosed as presenting with symptoms of neuropathic pain can be included in the study. These patients may have pure neuropathic pain, or mixed pain (neuropathic pain plus either somatic or visceral). Those presenting with somatic or visceral pain only would not be included at this time, although such patients can be added to other studies. Patients presenting with either meningitis or arachnoiditis can not be included in the study.

The treatment plan can be as follows:

- The patient's physician can determine if intrathecal opioids are contraindicated or unlikely to relieve the pain. If this is the case, the patient will be randomly assigned to receive either one of the three antisense doses or vehicle.

- The patient's physician determines that intrathecal opioids may relieve the pain. Intrathecal opioids are administered until their effectiveness is clear. If the opioids provide sufficient pain relief, the patient can be maintained on this treatment and not included in the study. If the opioids provide some but not adequate pain relief (as determined by the patient), the opioid infusion can be maintained and supplemented by either one of the three antisense doses or vehicle (randomly determined). If the opioids provide no noticeable pain relief, or relief only with intolerable side effects, the intrathecal opioid will be discontinued and the patient will be randomly assigned to receive either one of the three antisense doses or vehicle.

It is estimated that approximately 300 patients per year are treated for pain in the palliative care units. Of these, opioid therapy is problematic for 30%, and of these 30%, 2/3 have neuropathic pain. One example of a study group would contain eight patients per dose group for a total of 32 patients. Due to ethical considerations, patients must be maintained on their current pain and cancer treatments. A worker skilled in the art would realize that the effectiveness of the antisense may be influenced by concurrently prescribed medications. However, testing these interactions is not the focus of the study. The primary outcome measure is whether addition of antisense oligonucleotide to the treatment improves pain control. This is the first part of a larger study, and future projects can be performed to study drug interactions if the antisense proves to be an effective analgesic.

Assessment of patients prior to treatment

Prior to commencement of the study, several measures can be taken to first classify the patients. Patients can first be assessed, using the Edmonton Cancer Pain Staging and Classification System (Bruera *et al* (1989) *Pain* **37**: 203-209; Bruera *et al* (1995) *J Pain Sympt Manage* **10**: 348-355). The severity of pain currently being experienced by the patients are assessed using the numerical rating scale of average pain in the last 24 hours, as well as pain during rest and upon movement (from 0 = no pain to 10 = worst pain imaginable) from the Brief Pain Inventory (BPI; Daut *et al* (1983) *Pain* **17**: 197-210). Patients' overall quality of life can be assessed using the McGill Quality of Life Questionnaire (MQOL) (Cohen *et al* (1995) *Palliative Medicine* **9**: 207-219). The MQOL measures physical symptoms; physical, psychological and existential well-being; support; and overall quality of life. To assess symptoms other than pain (nausea, mood, appetite, insomnia, mobility, fatigue) we will use the Symptom Distress Scale (SDS) developed by McCorkle and Young (1978) *Cancer Nursing* **1**: 373-378. Performance status and overall motor function can be assessed using the Edmonton Functional Assessment Tool (EFAT) (Kaasa *et al* (1997) *J Pain and Sympt Manage* **13**: 10-19). The Folstein Mini-Mental State Examination (MMSE; Folstein *et al* (1975) *J Phychiatr Res* **12**: 189-198) can be used to assess cognitive function and short term memory. Sensitivity to hot and cold stimuli can be tested by recording patients' rating of intensity on a visual analog scale of thermal stimuli produced by a 9 cm^2 peltier-type contact thermode (Morin and Bushnell (1998) *Pain* **74**: 67-73). Cold temperatures can range from -5°C to 23°C, while heat

temperatures will range from 41°C to 48°C (Morin and Bushnell (1998) *Pain* 74: 67-73). Mechanical sensitivity can be assessed by calculating the 50% response threshold (in grams) to von Frey hair stimulation. These instruments have been chosen because they are quickly and easily administered, and present a minimal burden to the patient.

Intrathecal administration of drugs

Intrathecal administration of oligonucleotides is a preferred mode of delivery for two reasons. First, central administration allows the use of non-toxic phosphodiester-bonded oligonucleotides. Second, intrathecal administration of antisense oligonucleotides has been shown to be effective against neuropathic pain in rats. Moreover, patients can be approached for whom oral or subcutaneous administration of analgesics is ineffective, and who are scheduled to receive intrathecal treatment. Patients should continue with their normally scheduled analgesic therapy while in the study. Studies presented herein show that intrathecal morphine injection induces analgesia more effectively in antisense-treated neuropathic rats than in vehicle- or missense-treated neuropathic rats.

A range of doses of antisense oligonucleotides (0 (vehicle), 25 μg/day, 50 μg/day, and 100 μg/day; i.e. 4.167 μg/hr at the highest dose) can be tested. The pre-clinical trials presented herein indicate that a missense oligonucleotide, which is a control for antisense drug mechanism, has no behavioral or physiological effect. It is possible to exclude missense, because one placebo is already included (vehicle, i.e. artificial CSF), in the initial clinical trials if the lack of physiological effect is borne out by our pre-clinical toxicology and protein analysis studies. An exemplary dose range for antisense oligonucleotide treatment includes the dose (50 μg/day). The design of the study can be double blinded. Although as few as 4 subjects can be used to obtain reliable pharmacokinetic data (Glover *et al* (1997) *J Pharmacol Exp Ther* **282**: 1173-1180), 8 patients can be used per dose (for a total of 32 patients out of an available 60 per year).

Intrathecal catheterization of human patients are ideally performed by an anaesthetist. In an operating theatre with full aseptic precautions a polyesther catheter (Portex™) is inserted subarachnoidally. In detail, with the patient in a lateral position, lumbar puncture (18 G Tuohy

needle) is performed at an interspace between the second and the fifth lumbar vertebra using a lateral approach. Thereafter, the catheter is inserted 3-4 cm, and tunneled subcutaneously around the flank to the portal site over the lower ribs. The catheter can be fixed with a transparent self-adhesive dressing (Tegaderm™). All procedures should be performed using local anesthetic infiltration and intravenous sedation (e.g. fentanyl, diazepam) when needed. The catheter is connected to a computerized CADD infusion pump (Deltec™, Pharmacia) designed to give a continuous infusion of drug at a rate of 10 µl/hr (Gestin *et al* (1997) *Ann Fr Anesth Réanim* 5: 346-350) and the infusion can be started immediately. If clinical signs of arachnoiditis or meningitis are found, as monitored by the primary care nurse, a physician should be contacted immediately and the catheter removed and cultured for bacterial analysis. Patients who complete the study should be infused continuously with antisense oligonucleotide for 3 days (72 hours).

Pharmacokinetic monitoring

To fulfill the Phase I criteria of the study, distribution of the oligonucleotides should be monitored. Samples of CSF, blood and urine can be collected at regular intervals for chemical analysis. The pharmacokinetic data can be analyzed in collaboration with an expert clinical pharmacologist.

One CSF sample (1 ml) is collected prior to the commencement of the study. Subsequent 1 ml samples are collected 1 h, 6 h, 24 h, 48 h and 72 h after the initiation of drug infusion, then once per day until the pain returns up to a maximum of 1 week after cessation oligonucleotide treatment..

The oligonucleotides can be administered centrally and are, therefore, unlikely to enter systemic circulation. In addition, phosphodiester-bonded oligonucleotides are degraded quickly in blood. However, to be thorough, and to verify these assumptions, blood samples can be collected at regular intervals. Blood (10-15 ml) can be collected once prior to, and 2, 10, 15, 30, 60, 90, and 120 min, and 3, 6, 12, 24, 43 and 72 h after we begin drug infusion, and then once per day until the pain returns up to a maximum of 1 week after cessation oligonucleotide treatment.

To further verify that the oligonucleotides do not reach systemic circulation, and are not eliminated via the kidney, we will also collect urine samples at regular intervals. Urine samples will be collected prior to, and again 1 h, 6 h, 24 h, 48 h and 72 h after initiation of drug infusion, and then once per day until the pain returns up to a maximum of 1 week after cessation oligonucleotide treatment.

All samples should be collected, placed on dry ice immediately and transported to a freezer to be stored at –70 °C until analysis. Samples can be prepared using standard techniques known in the art and the amount of oligonucleotide present can be determined using HPLC with UV detection. Pharmacokinetic analysis can be performed as described for samples collected from rats.

Monitoring of patient outcome

Pain can be assessed using 0 – 10 numerical rating scales for average pain, pain at rest, and pain upon movement during the past 24 hours. These can be completed prior to infusion, and daily after the initiation of drug infusion for 10 days (i.e. the 3 days of drug infusion, plus 7 days after cessation of drug infusion). Pain can be assessed biweekly during weeks 2, 3 and 4 post-infusion, weekly thereafter until the pain returns up to a maximum of 13 weeks. Cognitive status (MMSE) and symptom distress (SDS) can be assessed whenever the numerical pain rating scales are completed. In addition, the complete BPI, EFAT, sensitivity to thermal and mechanical stimuli, and MQOL can be completed prior to and at 72 hour after initiation of infusion, one week after initiation of infusion, and weekly thereafter until pain returns up to a maximum of 13 weeks post-infusion.

The primary outcome measure can be the pain rating obtained with numerical rating scale of average pain in the past 24 hours. A two-way, one-repeated measure ANOVA ($\alpha = 0.05$) can be carried out with dose (independent variable with levels at 0, 25, 50 and 100 μg/day) and time (repeated measures factor at levels prior to infusion, and every 24 hours for the first 10 days following initiation of the infusion) as the factors. Tukey's Honestly Significant Difference test can be used for post-hoc analyses ($\alpha = 0.05$). The score for "average pain" at

72 hours after the initiation of the infusion can be categorized as mild (0-4), moderate (5-6) or severe (7-10) (Serlin *et al* (1995) *Pain* 61: 277-284) to determine the percentage of patients in each category at each dose level. Secondary measures can include symptoms and side effects as rated by the SDS, EFAT and MMSE. Patients can be assessed as to whether symptoms and side effects decreased, stayed the same or increased, compared to pre-treatment scores, during the course of oligonucleotide treatment. The MQOL can also be administered to evaluate the patients' overall satisfaction with care and quality of life. The optimum outcome is good pain relief, without an increase in symptoms and side effects, or cognitive or functional impairment.

To gain a better understanding of the invention described herein, the following examples are set forth. It should be understood that these examples are for illustrative purposes only. Therefore, they should not limit the scope of this invention in any way.

EXAMPLES

EXAMPLE I: ALLEVIATION OF PAIN AND RESTORATION OF OPIOID EFFICACY BY ANTISENSE KNOCKDOWN OF SPINAL MGLUR$_1$ IN RATS WITH NERVE INJURY

The effects of decreasing mGluR$_1$ receptor were demonstrated number with mGluR$_1$ AS oligonucleotide treatment in rats with a chronic constriction injury of one sciatic nerve. Specifically, spinal knockdown of mGluR$_1$ reduces cold and hot hyperalgesia and mechanical allodynia in neuropathic rats; knockdown of mGluR$_1$ restores morphine sensitivity, and reduces NMDA sensitivity in neuropathic rats; and enhanced PKC activity associated with nerve injury is reversed by knockdown of mGluR$_1$.

Materials and Methods

Subjects and surgery

Male Long Evans rats weighing 275-300 grams were used at the beginning of the experiment (Charles River, PQ). Rats were housed in groups of 3-4, with food and water available *ad libitum*, on a 12:12 hour light:dark cycle (lights on at 07:30). All surgical and testing procedures conformed to the ethical guidelines enforced by the Clinical Research Institute of

Montreal, and the Canadian Council on Animal Care. Nerve injury was induced by placing a 2 mm length of PE90 polyethylene tubing around one sciatic nerve (Mosconi & Kruger, (1996) *Pain* **64**: 37-57). Sham surgery consisted of exposing the nerve, but not placing a cuff. Intrathecal (i.t.) catheters were inserted using a lumbar catheterization method (Storkson *et al*, (1996) *J Neurosci Methods* **65**: 167-172). The catheter was attached to an Alzet osmotic mini-pump (ALZA Model 2001™) containing either artificial cerebrospinal fluid, antisense (AS) oligonucleotide solution, or missense (MS) oligonucleotide solution.

Oligonucleotides and drugs

An antisense (AS: 5'-GAG CCG GAC CAT TGT GGC-3') oligonucleotide was designed that is complementary to base pairs 371-388 of the rat $mGluR_1$ gene RATGPCR. A missense (MS: 5'-GAG CCG AGC ACT GTG TGC-3') oligonucleotide was designed by taking the AS sequence and mismatching four base pair couples. Oligonucleotides were purchased from Medicorp Inc (Montreal, QC). The vehicle used to dissolve the oligonucleotides, and as the vehicle treatment, was artificial cerebrospinal fluid (128.6 mM NaCl, 2.6 mM KCl, 1.0 mM $MgCl_2$, 1.4 mM $CaCl_2$, phosphate buffered to pH 7.4; ACSF). Vehicle, AS and MS were continuously infused i.t. for 7 days, via the catheter, in a volume of 1 µl hr -1. The daily dose of AS and MS was 50 µg day -1. This dose of AS and MS oligonucleotides was not found to produce any motoric or sedative side-effects, as examined using placing, righting and grasping reflexes. Morphine (Sabex™, QC) was dissolved in 0.9% saline, and injected intrathecally (i.t.), via lumbar puncture between vertebrae L4 and L5, in doses of 3, 10 or 30 µg in 20 µl .

Western blot analysis

Rats were quickly decapitated, and their spinal cords were pressure ejected and rapidly frozen. Spinal cords were stored at -70°C until analysis. The lumbar enlargement section of spinal cords were homogenized in Tris buffer containing protease inhibitors. Group I mGluRs are most likely found in the dorsal horn in lumbar spinal cord, with some expression in the intermediate gray matter and ventral horn (Hargett *et al*, (1998) *Soc Neurosci Abstr* **24**: 1869). Concentration of protein in each sample was determined using the method of Bradford (Bradford, (1976) *Anal Biochem* **72**: 248-254). The concentration of protein in each sample fell on the linear portion of the curve. For separation, 20 µg of total protein was loaded onto

the gel for electrophoresis. Proteins were separated by gel electrophoresis (SDS-PAGE; 7.5% polyacrylamide gel), and electrotransferred to PVDF membrane. The membrane was probed with an anti-rat $mGluR_1$ (primary antibody) raised in rabbits (Upstate Biotechnology, Lake Placid, NY). These antibodies are raised against the C termini of the receptor, a region that is unique to the receptor, and specificity was verified with immunoblotting (Martin *et al*, (1992) *Neuron* **9**: 259-270; Abe *et al*, (1992) *J Biol Chem* **267**: 13361-13368; Upstate Biotechnology). The primary antibody was later tagged with a peroxidase-conjugated anti-rabbit antibody (secondary antibody; Jackson Laboratories). The secondary antibody was detected by chemiluminescence (Boehringer Mannheim) and the membranes were apposed to Kodak Biomax MR film for one minute. Band density was measured using Alpha Imager software. The $mGluR_1$ is a protein of approximately 133 -142kD (Houamed *et al*, (1991) *Science* **252**: 1318-1321; Martin *et al*, (1992) *Neuron* **9**: 259-270; Masu *et al*, (1991) *Nature* **349**: 760-765).

Note that separate gels were run for each analysis ($mGluR_1$ in lumbar spinal cord at 7 days of oligonucleotide infusion; $mGluR_1$ in thalamus/PAG at 7 days of oligonucleotide infusion; $mGluR_1$ in lumbar spinal cord 12 days after cessation of oligonucleotide infusion). Since samples from vehicle-treated and oligonucleotide-treated rats were run on each gel, the relative change in amount of receptor protein in samples from oligonucleotide-treated rats were compared to vehicle-treated rats within each gel.

Spontaneous nociceptive behaviors

The ability of the selective group I mGluR agonist 3,5-dihydroxyphenylglycine (DHPG; Tocris Cookson, Ballwin, MO) to induce spontaneous nociceptive behaviors (SNBs) was assessed in a separate group of rats. Intrathecal catheters were implanted as described above, and rats were infused with either ACSF, or 50 μg day $^{-1}$ AS or MS oligonucleotides for 7 days. On the 7th day of treatment, rats were injected intrathecally via lumbar puncture with 50 nmol in 30 μl DHPG, and the time spent exhibiting SNBs over a 60 min observation period was recorded. SNBs included elevation of tail, licking of tail, elevation of hindpaws and licking of hindpaws. The time spent in each of these behaviors was combined into one "time spent exhibiting SNBs"

score. Data were analyzed by ANOVA followed by post-hoc Fisher's LSD t-tests on significant results.

The ability of intrathecal NMDA to induce pain behaviors was assessed in a separate group of nerve cuffed and sham-operated rats. NMDA (Sigma, Oakville, ON) was injected intrathecally (i.t.), via an i.t. catheter, in doses of 1.67 and 2.5 nmol in 20 µl. Rats were observed for a period of 8 min and the time spent exhibiting SNBs was recorded (time spent favoring paws, agitation, licking and biting paws).

Assessing neuropathic hyperalgesia and allodynia

In rats that were pre-treated with i.t. oligonucleotides, cold, hot and mechanical sensitivity were measured prior to any treatment (baseline) and again 4, 8, 12 and 16 days after nerve injury. In rats that were post-treated with i.t. oligonucleotides, cold, hot and mechanical sensitivity were measured prior to any treatment (baseline) and again 4 days after nerve injury (but before i.t. treatment), as well as 8, 12 and 18 days after nerve injury.

Cold sensitivity was measured by placing rats in a 1 cm deep 1 °C cold water bath for 75 sec, and counting the number of responses (lifting of hindpaw). Cold hyperalgesia was assessed by calculating the increase in number of responses, compared to baseline, from days 4 to 16 or 18 after nerve injury.

Mechanical sensitivity was measured by applying thin filaments (von Frey hairs) to the plantar surface of the hindpaw and determining the 50% response threshold (in grams) for paw withdrawal using the up-down method of filament presentation (Chaplan *et al*, (1994) *J Neurosci Methods* **53**: 55-63). Mechanical allodynia was assessed by calculating the percent decrease in 50% response threshold (from baseline) on days 4 to 16 or 18 after nerve injury.

Heat sensitivity was measured by applying focussed radiant heat to the glass under the plantar surface of the hindpaw and measuring the latency for the rat to withdraw its paw (Hargreaves *et al*, (1988) *Pain* **32**: 77-88). A cut-off latency of 20 sec was used to prevent tissue injury.

Heat hyperalgesia was assessed by calculating the percent decrease in latency (from baseline) on days 4 to 16 or 18 after nerve injury.

For tests of cold, mechanical and heat sensitivity, data from the pre-treatment schedule were analyzed by repeated measures ANOVA with nerve injury condition and i.t. treatment as independent groups factors, and days post nerve injury as a repeated measures factor. Data from the post-treatment schedule were analyzed by repeated measures ANOVA with i.t. treatment as the independent groups factor and days post nerve injury as the repeated measures factor. Significant results were further analyzed with post-hoc Fisher's LSD t-tests.

Morphine dose-response curve

To assess the analgesic effect of intrathecal (i.t.) morphine sulfate, rats were tested by measuring their latency to withdraw their tail from 55 °C water. A cut-off latency of 10 sec was used to prevent tissue injury. Tail flick latencies were measured prior to any treatment (naïve rats before oligonucleotide infusion or nerve injury; BL1), and again 4 days after nerve injury (after 7 days of oligonucleotide infusion; BL2). In ACSF-treated neuropathic rats, BL2 (2.44 ± 0.12) was slightly, but significantly higher than BL1 (2.01 ± 0.22) ($p < 0.05$), indicating that the rats were not hyperalgesic after sciatic nerve injury. There were no differences between BL1 and BL2 for any of the other treatment groups (ACSF sham BL1 = 2.01 ± 0.15 and BL2 = 2.19 ± 0.18; AS cuffed BL1 = 2.44 and BL2 = 2.46 ± 0.16; MS cuffed BL1 = 1.90 ± 0.17 and BL2 = 1.97 ± 0.18). There were no differences between oligonucleotide-treated rats and ACSF-treated rats for either BL1 or BL2. These results indicate that rats with a sciatic nerve injury were not hyperalgesic in the tail, replicating the results of Ossipov, *et al* ((1995) *Neurosci Lett* **199**: 83-86 and 87-90).

Furthermore, because BL1 and BL2 were not different in oligonucleotide-treated rats versus vehicle-treated rats, this suggests that antisense treatment did not affect motor function and the ability to respond on this test. After the measurement of BL2, rats were injected intrathecally with morphine in a dose of either 3, 10 or 30 μg per 20 μl via lumbar puncture between vertebrae L4 and L5. Following morphine injection, tail flick latencies were measured every 15 minutes from 15 to 60 minutes post-morphine. Latency scores were

converted to percent maximum possible effect scores [%MPE = ((latency – BL2) ÷ (cutoff – BL2)) × 100]. From the %MPE scores, we calculated the area under the curve (AUC) from 15 to 60 min post morphine injection (maximum AUC = 300) to clarify the analgesic effect. The ED_{50} was calculated with 95% confidence intervals from the AUC scores, using a regression of the log dose response curve, with confidence intervals calculated according to the method of Goldstein (Goldstein (1964) *Biostatistics: An Introductory Text*, The MacMillan Company, New York).

[^{3}H]PDBu binding

A [^{3}H]-phorbol-12,13-dibutyrate ([^{3}H]PDBu) binding assay (Olds *et al*, (1989) *Science* **245**: 866-869; Worley *et al*, (1986) *J Neurosci* **6**: 199-207) was used to demonstrate the amount of membrane-bound (i.e. activated) PKC. It has previously been shown that [^{3}H]PDBu binding is enhanced in spinal cord in rats with a sciatic nerve injury (Mao *et al*, (1992) *Brain Res* **588**: 144-149). Traditionally, it is assumed that PKC has to be transported to the membrane and bind DAG to be activated. Although this does not provide absolute quantification of PKC activity, it does provide some indication of the activation of PKC.

Four days after nerve injury (7 days of oligonucleotide infusion), rats were decapitated, and spinal cords quickly removed by pressure ejection and frozen at –70 °C. Serial transverse sections (20 μm) of lumbar spinal cord were cut at –18 °C and thaw mounted onto gelatin coated slides. Slides were dried overnight under a vacuum, and stored at –70 °C until autoradiographic processing. Sections were pre-incubated for 1 hr at 4 °C in buffer containing 50 mM Tris-HCl (pH 7.7), 100 mM NaCl and 1 mM $CaCl_2$. Sections were then incubated in buffer containing 0.1% bovine serum albumin and 2.5 nM [^{3}H]PDBu for 1 hr at 33 °C. Non-specific binding was assessed by adding excess unlabeled PDBu (1 μM). Computer images were generated and binding density was analyzed using the MCID image analysis system.

Treatment schedule

Pre-treatment: Three days prior to nerve injury, rats were implanted with i.t. catheters attached to mini osmotic pumps containing either vehicle, or 50 μg day $^{-1}$ AS or MS. Nerve injury was induced three days later, and rats were tested 4, 8, 12 and 16 days after nerve injury

for cold hyperalgesia, hot hyperalgesia and mechanical allodynia. Oligonucleotides were infused for a total of 7 days (from 3 days prior to until 4 days after nerve injury) . This pre-treatment schedule was employed to determine whether $mGluR_1$ is involved in the *development* of hyperalgesia and/or allodynia associated with nerve injury. Western blot analysis, [^{3}H]PDBu binding, morphine analgesia and NMDA sensitivity were assessed in rats with this treatment schedule (pre-treatment).

Post-treatment: A separate group of rats was used to determine whether $mGluR_1$ is involved in the *maintenance* of neuropathic pain, and whether AS oligonucleotide knockdown of $mGluR_1$ could *reverse* hyperalgesia and/or allodynia associated with an established neuropathy. Briefly, nerve injury was induced by placing a polyethylene cuff around one sciatic nerve as described above. Because there was no effect of oligonucleotide treatment in sham-operated rats when it was given as a pre-treatment, and would be expected to have the greatest effect, a sham-operated group was not included in the post-treatment test. Rats were confirmed to be neuropathic (i.e. display hot and cold hyperalgesia and mechanical allodynia as described below) four days after nerve injury. Five days after nerve injury, intrathecal catheters were implanted as described above, and rats were chronically infused with either ACSF, or 50 μg day -1 AS or MS oligonucleotides for 7 days (from 5 to 12 days post nerve injury). Hot, cold and mechanical sensitivity was measured again on days 8, 12 and 18 post nerve constriction (corresponding to days 3, 7 and 13 after beginning oligonucleotide infusion).

Results

Western blot analysis

Western blot analysis was performed to determine whether AS oligonucleotide treatment inhibited production of $mGluR_1$ protein in the lumbar spinal cord or the thalamus/periaqueductal gray region of rats 4 days after nerve constriction (7 days of oligonucleotide infusion). The present study demonstrates an increase in $mGluR_1$ protein in ACSF-treated neuropathic rats compared to ACSF-treated sham-operated rats (+45%). In neuropathic rats, intrathecal AS oligonucleotide treatment decreased $mGluR_1$ protein in lumbar spinal cord by 43.85 %, as compared to ACSF treatment (Figure 1A). In sham-operated rats, intrathecal AS oligonucleotide treatment decreased $mGluR_1$ protein by 39.22% compared to

ACSF-treated rats. In contrast, compared to ACSF treatment, MS oligonucleotide treatment did not decrease spinal $mGluR_1$ protein in either neuropathic or sham-operated rats (Figure 1A). By 12 days after oligonucleotide infusion was stopped, $mGluR_1$ protein had recovered in lumbar spinal cord of neuropathic AS-treated rats (percent difference in binding density compared to ACSF : AS = +22.11 ± 27.24%, and MS = - 6.77 ± 15.98%, t-test $p > 0.05$), indicating that the treatment was reversible. In the thalamus/periaqueductal gray region of the brain, AS treatment induced some decrease in $mGluR_1$ protein compared to ACSF treatment in both neuropathic (AS-treated –24.61% compared to ACSF-treated) and sham-operated (AS-treated –17.80% compared to ACSF-treated) rats, suggesting that some of the behavioural effects may be due to actions in the brain as well as in the spinal cord (Figure 1B). MS treatment induced negligible changes in brain $mGluR_1$ levels (Figure 1B). The spinal levels of $mGluR_5$ appeared to be slightly and negligibly decreased in neuropathic rats (AS-treated – 7.67% compared to ACSF-treated rats), and more pronouncedly decreased in sham-operated rats (AS-treated –25.63% compared to ACSF-treated) (Figure 1C). However, because the effect was so minimal in neuropathic rats compared to the effects on $mGluR_1$ protein, it is unlikely that a decrease in $mGluR_5$ contributed significantly to the behavioural effects seen in neuropathic rats.

DHPG-induced spontaneous nociceptive behaviours

To confirm that the *in vivo* $mGluR_1$ AS treatment produced a functionally relevant block of $mGluR_1$ in behaving animals, the effects of the $mGluR_1$ AS treatment was assessed on nociception induced by intrathecal injection of the selective group I mGluR agonist DHPG. DHPG produced spontaneous nociceptive behaviours (SNBs), including elevating, licking and biting of the hindpaws and tail. These nociceptive behaviours were significantly reduced in AS-treated, but not MS-treated rats (Figure 2), confirming that the AS treatment is capable of reducing nociception induced by mGluR1 receptor activation.

Cold sensitivity in neuropathic rats

Cold sensitivity was tested by counting the number of lifts of the injured (ipsilateral) hindpaw when rats were placed in 1 cm deep water at 1 °C for 75 sec. Pre-treated ACSF- and MS-treated neuropathic rats exhibited a large increase from baseline in response frequency (Figure 3a) compared to sham-operated rats, on days 4 to 16 after nerve injury. Pre-treated AS-

treated neuropathic rats showed a significantly lower increase in response frequency (Figure 3a) in the cold water test, compared to ACSF- and MS-treated rats, and were not significantly different from sham-operated rats. There were no differences between i.t. treatment groups in sham-operated rats. There were also no differences between treatment groups in the contralateral paw (data not shown). When i.t. oligonucleotides were given as a post-treatment, all nerve-injured (neuropathic) rats displayed cold hyperalgesia four days after nerve injury, prior to i.t. infusion, as indicated by a large increase in response frequency (Figure 3b) of the ipsilateral paw, compared to baseline. On days 8, 12 and 18 after nerve injury (3, 7 and 13 days after beginning i.t. drug infusion) ACSF- and MS-treated neuropathic rats still exhibited a large increase from baseline in response frequency (Figure 3b) of the ipsilateral paw. In contrast, the increase in response frequency (Figure 3b) of the ipsilateral paw was significantly reduced in AS-treated neuropathic rats after i.t. treatment (Figure 3b). There were no differences between groups in the contralateral paw (data not shown).

Mechanical sensitivity in neuropathic rats

Mechanical sensitivity was assessed by determining the 50% response threshold, in grams, to stimulation of the plantar surface of the hindpaw with von Frey hairs. In the pre-treatment group, ACSF- and MS-treated neuropathic rats displayed a large decrease from baseline in 50% response threshold to von Frey hair stimulation of the ipsilateral hindpaw, as compared to sham-operated rats (Figure 3c). AS-treated neuropathic rats showed a significantly smaller decrease in response threshold, compared to ACSF- and MS-treated neuropathic rats, across test days, and were not different from sham-operated rats (Figure 3c). There were no differences between i.t. treatment groups in sham-operated rats (Figure 3c), or in the contralateral hindpaw (data not shown).

Similarly, in rats given post-nerve injury i.t. oligonucleotide infusion, all nerve injured rats displayed mechanical allodynia, as indicated by a large decrease in 50% response threshold in the ipsilateral hindpaw four days after nerve injury, prior to oligonucleotide infusion (Figure 3d). On days 8, 12 and 18 after nerve injury ACSF- and MS-treated neuropathic rats still exhibited a large decrease in 50% response threshold of the ipsilateral paw (Figure 3d). In contrast, the decrease in 50% response threshold in the ipsilateral hindpaw of AS-treated

neuropathic rats was significantly lower than in ACSF- or MS-treated rats (Figure 3d) after i.t. oligonucleotide treatment. There were no differences between groups in the contralateral paw (data not shown).

Heat sensitivity in neuropathic rats

Heat sensitivity was measured by stimulating the plantar surface the hindpaw with a focused radiant heat source and measuring the latency of the rats to withdraw the paw. In the pre-treatment group, ACSF- and MS-treated neuropathic rats displayed a large decrease from baseline in withdrawal latency to radiant heat stimulation of the ipsilateral paw, as compared to sham-operated rats (Figure 3e). In AS-treated neuropathic rats this decrease was significantly less, compared to ACSF- and MS-treated neuropathic rats (Figure 3e). There were no differences between i.t. treatment groups in sham-operated rats (Figure 3e), or in the contralateral hindpaw (data not shown).

Similarly, in rats given post-nerve injury i.t. drug infusion, all nerve injured rats displayed heat hyperalgesia, as indicated by a large decrease in response latency in the ipsilateral hindpaw four days after nerve injury, prior to oligonucleotide infusion (Figure 3f). On days 8, 12 and 18 after nerve injury ACSF- and MS-treated neuropathic rats still exhibited a large decrease in response latency of the ipsilateral paw (Figure 3f). In contrast, the decrease in response latency in the ipsilateral hindpaw of AS-treated neuropathic rats was significantly lower than in ACSF- or MS-treated rats (Figure 3f) after i.t. treatment. There were no differences between groups in the contralateral paw (data not shown).

Morphine dose-response curve

Four days after injury, ACSF- and MS-treated neuropathic rats displayed a minimal analgesic response to intrathecal morphine in the tail withdrawal test (Figure 4) compared to sham-operated rats, which responded normally to intrathecal morphine. In contrast, AS-treated neuropathic rats displayed significant morphine analgesia, and were not different from sham-operated rats (Figure 4), indicating that AS treatment restored opioid sensitivity in neuropathic rats. Note that, as mentioned in the methods section, the post-treatment baseline (prior to the i.t. injection of morphine) was not different from the pre-treatment baseline (prior to any i.t. infusion or nerve injury), therefore sciatic nerve injury did not cause hyperalgesia in the tail.

This demonstrates that AS treatment restored opioid sensitivity, rather than merely reversing hyperalgesia.

[^{3}H]PDBu binding

Figure 5A is a histogram summary of [^{3}H]PDBu binding density from all the slides in each treatment group, while Figure 5B is a computer-generated image showing [^{3}H]PDBu binding in a single representative slide from each treatment group. Because $mGluR_1$ is positively coupled to PI hydrolysis, and thus to activation of PKC, the involvement of PKC in neuropathic pain was examined by measuring the binding of [^{3}H]phorbol,12,13-dibutyrate ([^{3}H]PDBu) in lumbar spinal cord slices of rats four days after nerve constriction (7 days of oligonucleotide infusion). When activated, PKC has been shown to migrate to the cellular membrane, and [^{3}H]PDBu binds to the diacylglycerol site of PKC once it has been translocated to the membrane (Olds *et al*, (1989) *Science* **245**: 866-869; Worley *et al*, (1986) *J Neurosci* **6**: 199-207). [^{3}H]PDBu binding was significantly increased in lumbar spinal cord dorsal horn from ACSF- and MS-treated rats with nerve injury, as compared to sham-operated rats (Figure 5). In contrast, in lumbar spinal cord dorsal horn from AS-treated neuropathic rats, [^{3}H]PDBu binding was significantly lower compared to ACSF and MS-treated neuropathic rats, and was not different from binding seen in spinal cords from sham-operated rats (Figure 5). Although the representative slide from an AS-treated sham-operated rats appears to show reduced [^{3}H]PDBu binding compared to the slides from an ACSF- and MS-treated sham-operated rats, statistical analysis of all the data indicated no significant difference between the sham-operated groups.

NMDA-induced spontaneous nociceptive behaviours

It was found that ACSF- and MS-treated rats with nerve injury displayed increased nociceptive behaviour in response to intrathecal injection of NMDA, compared to sham-operated rats (Figure 6). However, AS oligonucleotide treatment significantly reduced the nociceptive behaviours induced by intrathecal injection of NMDA in nerve injured rats (Figure 6).

Discussion

Intrathecal infusion of our AS oligonucleotide greatly decreased $mGluR_1$ protein in lumbar spinal cord, and slightly decreased it in the brain. Further, using 12 days post-infusion, the amount of $mGluR_1$ protein in AS-treated neuropathic rats had recovered to levels similar to ACSF-treated rats, indicating that the effect was reversible. As a functional correlate of a decrease in $mGluR_1$ protein, it was shown that AS-treated rats displayed significantly fewer DHPG-induced SNBs. Moreover, AS oligonucleotide treatment significantly decreased cold hyperalgesia, mechanical allodynia and heat hyperalgesia of the ipsilateral hindpaw of neuropathic rats. The attenuation of neuropathic pain in $mGluR_1$ AS-treated rats was accompanied by a concomitant decrease in activated PKC, as indicated by a decrease in [^{3}H]PDBu binding. In relation to the possible common etiology of neuropathic pain and opioid tolerance/dependence, it is important to point out here that inhibition of both $mGluR_1$ and PKC activity in the brain has been shown to attenuate the precipitated morphine withdrawal syndrome (Fundytus and Coderre (1994) *Br J Pharmacol* **113**: 1215-1220; Fundytus and Coderre (1996) *Eur J Pharmacol* **300**: 173-181; Fundytus and Coderre (1999) *Pain Forum* **8(1)**: 3-13; Fundytus and Coderre (1999) *Pain Forum* **8(2)**: 59-63). Furthermore, spinal PKCγ is translocated to the membrane in neuropathic rats (Mao *et al*, (1995) *Neurosci Lett* **198**: 75-78), inhibition of PKC in the spinal cord reduces morphine tolerance and neuropathic pain (Mao *et al*, (1995) *Pain* **62**: 259-274; Mao *et al*, (1992) *Brain Res* **588**: 144-149), and PKCγ knockout mice do not develop neuropathy (Malmberg *et al*, (1997) *Science* **278**: 279-283). The activation of PKC may underlie the effects seen with inhibition of either $mGluR_1$ or NMDA receptors.

Clinically, neuropathic pain is often difficult to treat, being only partially relieved by high doses of opioids (Cherny *et al* (1994) *Neurology* **44**: 857-861; MacDonald (1991) *Recent Results in Cancer Res* **121**: 24-35; McQuay *et al* (1992) *Anesthesia* **47**: 757-767). Using this model of neuropathy, ACSF- and MS-treated neuropathic rats were shown to be less sensitive to the analgesic effects of intrathecally administered morphine, while $mGluR_1$ AS-treated neuropathic rats displayed a normal analgesic response to morphine. Thus, knockdown of $mGluR_1$ at the spinal level prevented the development of morphine insensitivity in neuropathic rats.

Activation of group I mGluRs has been shown to enhance activity at NMDA receptors (Bleakman *et al* (1992) *Mol Pharmacol* **42**: 192-196) via a PKC-mediated mechanism (Chen and Huang (1992) *Nature* **356**: 521-523; Harvey and Collingridge (1993) *Br J Pharmacol* **109**: 1085-1090; Raymond *et al* (1994) *J Physiol Paris* **88**: 181-192), and NMDA receptors have been shown to contribute to nociception in animal models of persistent nociception (Chaplan *et al* (1997) *J Pharmacol Exp Ther* **280**: 829-838; Mayer *et al* (1995) . *NIDA Res Mongr* **147**: 269-298; Yamamoto and Yaksh (1992) *Pain* **49**: 121-128). Based on the results from [^{3}H]PDBu binding, it was expected that NMDA receptor activity would be enhanced in neuropathic rats, and that neuropathic rats would therefore be more sensitive to the excitatory effects of NMDA injected intrathecally. It was also predicted that mGluR$_1$ AS oligonucleotide treatment would attenuate this enhanced NMDA receptor activity, and thus NMDA sensitivity, in neuropathic rats. In experiments designed to test these predictions, it was demonstrated that neuropathic rats with nerve injury displayed increased nociceptive behaviours in response to intrathecal injection of NMDA, and that this effect was reversed by mGluR$_1$ AS oligonucleotide treatment. Although it is possible that mGluR$_1$ AS treatment induced a reduction in the number of NMDA receptors, this is highly unlikely. First, unmodified, phosphodiester bonded oligonucleotides were used, which have generally been shown not to have non-sequence-specific effects. Moreover, if mGluR$_1$ AS treatment reduced the number of NMDA receptors, the response to i.t. NMDA would likely be blunted in the AS-treated rats compared to ACSF-treated sham-operated rats. However, the time spent exhibiting nociceptive behaviours was virtually identical for the two groups of rats. Thus, in this model of neuropathy, rats with nerve injury were confirmed to be more responsive to spinal administration of NMDA, and this could be attributed to mGluR$_1$-associated mechanisms.

Although profound effects were observed in neuropathic rats, mGluR$_1$ AS oligonucleotide treatment had no significant effect in sham-operated rats or in the contralateral paw of neuropathic rats, indicating that mGluR$_1$ is involved in chronic neuropathic pain, but not in the mediation of acute nociceptive stimuli. This is in agreement with a previous study where the blockade of mGluR$_1$ with selective antibodies alleviated cold hyperalgesia in neuropathic rats, but had no effect on response latency to focused radiant heat in naive rats, nor on formalin-induced pain scores (Fundytus *et al* (1998) *Soc Neurosci Abstr* **23**: 1013). However, this is in

contrast to other investigators who found that antisense knockdown of $mGluR_1$ increased response latency in a tail flick test in normal rats (Young *et al* (1998) *J Neurosci* **18**: 10180-10188). This discrepancy may be due to the differences in oligonucleotides used. In the present application unmodified, phosphodiester (PO)-bonded oligonucleotides were used, which have been shown to be non-toxic in the central nervous system even at very high concentrations, much higher than what we used (Akhtar and Agrawal (1997) *Trends Pharmacol Sci* **18**: 12-18; Whitesell *et al* (1993) *Proc Natl Acad Sci USA* **90**: 4665-4669). Although PO oligonucleotides are quickly degraded in blood, they have been shown to be stable in CSF (Akhtar and Agrawal (1997) *Trends Pharmacol Sci* **18**: 12-18; Wahlestedt (1994) *Trends Pharmacol. Sci.* **15**: 42-46; Whitesell *et al* (1993) *Proc Natl Acad Sci USA* **90**: 4665-4669; Yaida and Nowak (1995) *Regul Pept* **59**: 193-199). Young and colleagues used phosphorothioate (PS) modified oligonucleotides, which have been shown to be toxic in the central nervous system (CSF (Akhtar and Agrawal (1997) *Trends Pharmacol Sci* **18**: 12-18; Whitesell *et al* (1993) *Proc Natl Acad Sci USA* **90**: 4665-4669). Moreover, sequence specificity of the AS oligonucleotide of the presentstudy was demonstrated by using an MS control that was a re-arrangement of the exact same bases that were in our AS oligonucleotide (Wahlestedt (1994) *Trends Pharmacol. Sci.* **15**: 42-46; Phillips and Gyurko (1995) *Regul Pept* **59**: 131-141), thus preserving the GC content, and therefore physical properties of the oligonucleotide such as melting temperature and charge. Young and colleagues used a mismatch control in which purines had been substituted for pyrimidines, potentially altering the physical properties of the oligonucleotide. Thus, it is unclear whether the effects that Young and colleagues obtained were sequence-specific, or whether they were due to some combination of the PS modification and physical properties of the oligonucleotide. Further research is necessary to explore the nature of the discrepancies in the effects of knockdown of $mGluR_1$. Data from other investigators support our contention that mGluRs are involved in chronic rather than acute pain, and they show that antagonism of mGluRs does not attenuate the hyperalgesia associated with an incision injury in rats that mimics acute post-operative pain (Zahn and Brennan (1998) *Anesthesia & Analgesia* **87**: 1354-1359). In another recent study, Neugebauer *et al* (1994) showed that antagonism of group I mGluRs decreases responses in inflamed knee joint, but has no effect in normal animals.

In the current study, the data indicates a role for $mGluR_1$ in neuropathic pain. Because knockdown of $mGluR_1$ reverses nerve-injury induced insensitivity to morphine, as well as reducing neuropathic pain, the knockdown of $mGluR_1$ will be useful as therapy for neuropathic pain in the clinic. It may be used to alleviate pain directly, or as an adjunct to opioid analgesic therapy.

EXAMPLE II: ANTISENSE KNOCKDOWN OF MGLUR$_1$ REVERSES HYPERALGESIA/ALLODYNIA ASSOCIATED WITH AN ESTABLISHED NEUROPATHIC INJURY IN RATS

Subjects

Long Evans rats were used in this demonstration, weighing 300-350 grams at the time of surgery. Rats were housed 3-4 per cage, on a 12:12 hour light:dark cycle, with food and water available *ad libitum.* All procedures were approved by the Clinical Research Institute of Montreal Animal Care Committee.

Oligonucleotides

An antisense (AS) oligonucleotide (AS: 5'-GAG CCG GAC CAT TGT GGC-3') was designed to be complementary to base pairs 371-388 of the rat mGluR1 gene, RATGPCR. A control missense (MS) oligonucleotide was designed that had exactly the same bases as the AS sequence, with four base pairs mismatched (MS: 5'-GAG CCG AGC AC5 GTG TGC-3').

Surgery

Rats were rendered neuropathic by placing a 2 mm length of PE90 polyethylene tubing around one sciatic nerve (Mosconi and Kruger (1996) *Pain* **64**: 37-57). Intrathecal (i.t.) catheters, attached to model 2001 Alzet osmotic mini-pumps (ALZA Corp, Palo Alto, CA), were implanted five days after nerve injury. Rats were continuously infused for 7 days (from days 5 to 12 after nerve injury), with either artificial CSF (ACSF), or 50 μg/day AS or MS oligonucleotides.

Mechanical sensitivity

Mechanical sensitivity was measured as described in Example I. Testing was performed prior to any surgery or treatment (baseline), 4 days after nerve injury, and 8, 12 and 18 days after nerve injury. Mechanical allodynia was assessed by calculating the percent decrease in 50% response threshold compared to baseline.

Heat sensitivity

Heat sensitivity was measured, as described in Example I, prior to any surgery or treatment (baseline), 4 days after nerve injury, and 8, 12 and 18 days after nerve injury. Heat hyperalgesia was assessed by calculating the percent decrease in response latency compared to baseline.

Cold sensitivity

Cold sensitivity was measured as described in Example I. Testing was performed prior to any surgery or treatment (baseline), 4 days after nerve injury, and 8, 12 and 18 days after nerve injury. Cold hyperalgesia was assessed by calculating the increase in response frequency compared to baseline.

Results

Figure 7 depicts mechanical sensitivity in neuropathic rats treated with AS, MS or ACSF. Four days after nerve injury, prior to intrathecal infusion of oligonucleotides, all rats displayed mechanical allodynia as indicated by a large decrease in 50% response threshold from baseline. Drug infusion began on day 5 after nerve injury. From days 8 to 18 after nerve injury (3 to 13 days after initiation of oligonucleotide infusion) ACSF and MS treated rats were still allodynic, as indicated by a continued large decrease in 50% response threshold. In contrast, mechanical allodynia was reduced in AS treated rats, as indicated by an attenuated reduction in 50% response threshold.

Figure 8 shows heat sensitivity in neuropathic rats treated with AS, MS or ACSF. Four days after nerve injury, prior to intrathecal infusion of oligonucleotides, all rats displayed hot hyperalgesia, as indicated by a large decrease in response latency. Drug infusion began on day

5 after nerve injury. From days 8 to 18 after nerve injury, ACSF and MS treated rats continued to display hot hyperalgesia. In contrast, hot hyperalgesia was reversed in AS treated rats, as indicated by an attenuated reduction in response latency.

Figure 9 illustrates the cold sensitivity in neuropathic rats treated with AS, MS or ACSF. Four days after nerve injury, prior to drug infusion, all rats displayed cold hyperalgesia, as indicated by a large increase in the number of responses in the cold water bath. Drug infusion began on day 5. From days 8 to 18 after nerve injury, ACSF and MS treated continued to display cold hyperalgesia. Conversely, cold hyperalgesia was reversed in AS treated rats, as indicated by a reduced frequency of responding.

Discussion

In the present study it has been demonstrated that antisense oligonucleotide knockdown of $mGluR_1$ *reverses* the mechanical allodynia, hot and cold hyperalgesia associated with nerve injury in rats. These results suggest that $mGluR_1$ is involved in the *maintenance* of neuropathic pain. Previous results, showing a reduction in allodynia and hyperalgesia in nerve injured rats with antisense oligonucleotide pretreatment, indicated that $mGluR_1$ is involved in the *development* of neuropathic pain (Fundytus *et al*, (1997) *Soc. Neurosci. Abstr.* **23**: 1013). Previous studies also showed that antisense oligonucleotide knockdown of $mGluR_1$ restored opioid sensitivity in neuropathic rats (Fundytus *et al*, (1998) *INRC'98*, 42). Reduction of $mGluR_1$ protein in lumbar spinal cord, following intrathecal $mGluR_1$ antisense oligonucleotide treatment, was verified by Western blot analysis. Using a [^{3}H]phorbol 12,13 dibutyrate ([^{3}H]PDBu) binding assay, a functional reduction of $mGluR_1$ was also demonstrated by showing that enhanced PKC activity associated with neuropathic pain was attenuated by $mGluR_1$ antisense oligonucleotide treatment. Thus, $mGluR_1$ is a viable new target for drug development in the treatment of neuropathic pain.

EXAMPLE III: ANTISENSE KNOCKDOWN OF MGLUR$_1$ ALLEVIATES HYPERALGESIA DUE TO CHRONIC INFLAMMATION IN RATS

This example demonstrates that antisense oligonucleotide knockdown of spinal mGluR$_1$ significantly reduces heat hyperalgesia and mechanical allodynia associated with complete Freund's adjuvant induced chronic inflammation of one hindpaw.

Methods

Subjects, surgery and induction of inflammation

Subjects were male Wistar rats weighing 325-375 grams at the beginning of the experiment. Rats were housed 3 to 4 per cage with food and water freely available, with a 12:12 hour light:dark cycle (lights on at 06:00 hours).

Intrathecal (i.t.) catheters were inserted using a lumbar catheterization method (Storkson, Kjorsvik, Tjolsen and Hole, 1996). The catheter was attached to an Alzet osmotic mini-pump (ALZA Model 2001) containing either ACSF, antisense (AS) oligonucleotide solution, or missense (MS) oligonucleotide solution. Rats were infused intrathecally for 7 days.

Chronic unilateral inflammation was induced by transdermally injecting 25 µl of complete Freund's adjuvant (CFA) (Sigma) in each of the dorsal and ventral surface of one hindpaw.

Oligonuceotides

An antisense (AS: 5'-GAG CCG GAC CAT TGT GGC-3') oligonucleotide was designed that was complementary to base pairs 371-388 of the rat mGluR$_1$ gene RATGPCR. A missense (MS: 5'-GAG CCG AGC ACT GTG TGC-3') oligonucleotide was designed by taking the AS sequence and mismatching four base pair couples. Oligonucleotides were purchased from Medicorp Inc (Montreal, PQ). The vehicle used to dissolve the oligonucleotides, and as the vehicle treatment, was artificial cerebrospinal fluid (128.6 mM NaCl, 2.6 mM KCl, 1.0 mM $MgCl_2$, 1.4 mM $CaCl_2$, phosphate buffered to pH 7.4; ACSF). Vehicle, AS and MS were continuously infused i.t., via the catheter, in a volume of 1 µl/hr. The daily dose of AS and MS was 50 µg/day. Effective knockdown of receptors has been achieved with doses as low as 1 µg/day, up to doses as high as 720 µg/day (Wahlestedt, 1994). This dose of AS and MS

oligonucleotides was not found to produce any motoric or sedative side-effects, as examined using placing, righting and grasping reflexes.

Treatment schedule

Pre-treatment: Three days before injection of CFA rats were implanted with intrathecal catheters attached to osmotic mini-pumps containing either ACSF, AS or MS solution. Heat and mechanical sensitivity was measured prior to any surgery or injection (baseline), and again 1, 2, 4, 6 and 8 days after CFA injection. This treatment schedule was employed to see if $mGluR_1$ is involved in the development of inflammatory pain. Western blot analysis was carried out on lumbar spinal cords from rats in this treatment group.

Post-treatment: A separate group of rats was injected with CFA, followed by implantation of intrathecal catheters attached to osmotic mini-pumps containing either ACSF, AS or MS solution 2 days later. Heat and mechanical sensitivity was measured prior to any surgery or injection (baseline), and again 1, 2, 4, 6 and 8 days after CFA injection. This treatment was employed to determine if $mGluR_1$ was involved in the maintenance of inflammatory pain, and whether antisense oligonucleotide knockdown of spinal $mGluR_1$ could reverse pain due to an established inflammatory injury.

Assessment of heat hyperalgesia and mechanical allodynia

Heat sensitivity was measured by applying focussed radiant heat to the plantar surface of the hindpaw and measuring the latency for the rat to withdraw its paw (Hargreaves *et al* (1988) *Pain* **32**: 77-88). Heat hyperalgesia was assessed by calculating the percent decrease in latency (from baseline) on days 1 to 8 after CFA injection. Data were analyzed by repeated measures ANOVA with i.t. treatment as the independent groups factor and days post CFA injection as the repeated measures factor. Significant results were further analyzed with post-hoc Fisher's LSD t-tests.

Mechanical sensitivity was measured by applying thin filaments (von Frey hairs) to the plantar surface of the hindpaw and determining the 50% response threshold (in grams) for paw withdrawal using the up-down method of filament presentation (Chaplan *et al* (1994) *J. Neurosci. Methods* **53**: 55-63). Mechanical allodynia was assessed by calculating the percent

decrease in 50% response threshold (from baseline) on days 1 to 8 after CFA injection. Data were analyzed by repeated measures ANOVA with i.t. treatment as the independent groups factor and days post CFA injection as the repeated measures factor. Significant results were further analyzed with post-hoc Fisher's LSD t-tests.

Western blot analysis (protein determination)

The Western blot analysis was performed as described in the previous example except that the proteins were separated by polyacrylamide gel electrophoresis using a 5% polyacrylamide gel rather than a 7.5% polyacrylamide gel.

Results

Heat hyperalgesia and mechanical allodynia

In rats pre-treated with vehicle or oligonucleotides, ACSF and MS-treated rats displayed significant heat hyperalgesia from days 1 to 8 after CFA injection, as indicated by a large decrease from baseline in response latency (Figure 10A) when focussed radiant heat was applied to the ventral surface of the injected paw. Although there was no effect of oligonucleotide treatment on day 2 after CFA injection, heat hyperalgesia was attenuated in AS-treated rats as indicated by longer response latencies on days 4 to 8 after CFA injection (Figure 10A).

In the post-treatment group, all rats displayed heat hyperalgesia, as indicated by a large decrease from baseline in response latency (Figure 10B) on day 1 after CFA injection, prior to the infusion of oligonucleotides. Oligonucleotide infusion began on day 2 after CFA injection. ACSF and MS treated rats remained hyperalgesic from days 4 to 8 after CFA injection, as indicated by a continued reduction in response latency in the injected paw. In contrast, heat hyperalgesia was attenuated in AS treated rats, as indicated by an increase in response latency following drug infusion on days 4 to 8 after CFA injection (Figure 10B).

In rats pre-treated with vehicle or oligonucleotides, ACSF and MS treated displayed significant mechanical allodynia on days 1 to 8 after CFA injection, as indicated by a large decrease from baseline in 50% response threshold (grams) in response to von Frey hair

stimulation of the plantar surface of the injected paw (Figure 11A). Mechanical allodynia was attenuated in AS treated rats as indicated by higher 50% response thresholds (Figure 11A).

In the post-treatment group, all rats displayed mechanical allodynia on day 1 after CFA injection, prior to oligonucleotide infusion, as indicated by a large decrease in 50% response threshold compared to baseline (Figure 11B). Following oligonucleotide infusion, ACSF and MS treated rats remained allodynic from days 4 to 8 after CFA injection, as indicated by a continued large decrease from baseline 50% response threshold (Figure 11B). In contrast, mechanical allodynia was reversed in AS treated rats, as indicated by significantly higher 50% response thresholds on days 4 to 8 after CFA injection (Figure 11B).

Western blot analysis

Western blot analysis showed that in ACSF- and MS-treated, CFA-injected rats there was a slight increase (+20.55%) in $mGluR_1$ protein in lumbar spinal cord compared to naïve rats. There was no difference in $mGluR_1$ protein between ACSF- and MS- treated rats (MS-treated versus ACSF-treated = -3.93%) (Figure 11). In contrast, the amount of $mGluR_1$ protein was greatly decreased in lumbar spinal cords of AS-treated, CFA-injected rats compared to either naïve rats (-52.46%) or ACSF-treated, CFA-injected rats (-60.56%) (Figure 11).

Discussion

This example demonstrates that antisense oligonucleotide knockdown of spinal $mGluR_1$ reduces heat hyperalgesia and mechanical allodynia associated with CFA-induced chronic inflammation. It in interesting to note that in rats pre-treated with $mGluR_1$ AS oligonucleotide, a reduction in heat hyperalgesia was not observed until 4 days after CFA injection. This suggests that group I mGluRs may be more important in the mediation of chronic, rather than acute pain. This is in agreement with previous work from the applicant's laboratory. An earlier study showed that whereas i.t. administration of antibodies selective for $mGluR_1$ reduced chronic neuropathic pain, there was no effect on heat sensitivity in naïve rats, or on pain scores induced by short-term formalin-induced inflammation (Fundytus *et al* (1998) *Soc Neurosci Abst* **23**:1013). Recent work examining the effect of knockdown of spinal $mGluR_1$ in neuropathic pain also supports this suggestion. Although knockdown of spinal $mGluR_1$ reduced hyperalgesia and allodynia in the hindpaw ipsilateral to a sciatic nerve

constriction, there was no significant effect in either the contralateral paw or in sham-operated animals (Fundytus *et al* (2000) submitted). However, mechanical allodynia was significantly reduced by AS treatment at all time points. These results suggest that perhaps the mechanisms by which $mGluR_1$ mediates heat and mechanical sensitivity in models of inflammatory pain may be different, while the mechanisms by which thermal and mechanical sensitivity are mediated in neuropathic pain may be similar.

EXAMPLE IV: TOXICITY IN HUMAN CELL CULTURE

To test for toxicity, the effects of oligonucleotides in cultures of a non-tumorigenic human cell line that contains group I mGluRs, for example SH-SY5Y, obtained from American Type Culture Collection (ATCC, Rockland, MD) (Lee *et al* (1995) *Proc Natl Acad Sci USA* **92**: 8083-8087), were monitored. Cells were grown to confluency in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 µg/ml streptomycin (all from Sigma, St. Louis, MO), in monolayers in plastic Corning 150 cm^2 culture flasks (Sigma) at 37 °C, under 5% CO_2/95% air. Medium was changed three time per week, and cells pack-cultured every 5-7 days to maintain the cell line. The growth media was decanted, and the cells were rinsed with 6 ml of 0.02% EDTA, which was not discarded. Another 6 ml of 02% EDTA was added, and the cells were incubated at 37 °C for 30 minutes. The cells were counted on Neubauer hemacytometer (Fisher Scientific, Orlando, FL), and re-suspended in growth media at a density of 1.0×10^6 cells/ml. Experiments were initiated by back-culturing. Cells were suspended and centrifuged at 1000 rpm for 5 min. The cell pellet was re-suspended in the treatment medium at a concentration of 1.0×10^6 cells/ml, and plated at 0.25 ml/well (low density plating) in 24 well Falcon plates (Fisher Scientific). Treatment media was growth media with either antisense or missense oligonucleotide (dissolved in artificial CSF) added in a concentration of 0.2 to 20 mM, preferrably 10 µM. Because of the seriousness of this application (human treatment) higher concentrations were tested than what would likely be used in either rat or human CSF for oligonucleotide treatment. In rats, the highest dose was 1000 µg/day. If there is absolutely no degradation of oligonucleotides, the maximum concentration reached at the end of the 14 day infusion would be 14000 µg/2000 µl of CSF (a rat has approximately 2 ml of CSF). The molecular weight of oligonucleotide

obtained from Upstate Biotechnology is 5813.9 g/mol. Therefore, the maximum concentration for CSF in the rat was 1.20 x 10^{-3} μM (or 1.20 x 10^{-6} mM). Cell viability was assessed at 24, 48, 72 and 168 hours of treatment using the trypan blue dye exclusion method. Trypan blue dye is excluded from live cells with intact membranes, but stains dead cells. The cells were counted in a hemocytometer (see above) with a long-distance phase contrast microscope. Live cells were clear and dead cells were blue. Oligonucleotide treated cell cultures were compared to non-treated cultures, and artificial CSF treated cultures to determine the percentage of cells which died within each time period. Unacceptable toxicity is indicated by >20% more cell death in oligonucleotide treated cells relative to controls.

The resulting growth curves and percent mortality data are presented in Figures 12 - 18. None of the antisense sequences of the present invention cause a decrease in cell growth in comparison to cells treated with the vehicle alone. Similarly, this antisense treatment did not result in significant cell mortality.

EXAMPLE V: EFFICACY IN CELL CULTURE

To test for efficacy, the effects of oligonucleotides in cultures of a non-tumorigenic human cell line that contains group I mGluRs, for example SH-SY5Y, obtained from American Type Culture Collection (ATCC, Rockland, MD) (Lee *et al* (1995) *Proc Natl Acad Sci USA* **92**: 8083-8087), were monitored. Cells were grown to confluency in RPMI-1640 media supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, and 100 μg/ml streptomycin (all from Sigma, St. Louis, MO), in monolayers in plastic Corning 150 cm^2 culture flasks (Sigma) at 37 °C, under 5% CO_2/95% air. Medium was changed three time per week, and cells pack-cultured every 5-7 days to maintain the cell line. The growth media was decanted, and the cells were rinsed with 6 ml of 0.02% EDTA, which was not discarded. Another 6 ml of 02% EDTA was added, and the cells were incubated at 37 °C for 30 minutes. The cells were counted on Neubauer hemacytometer (Fisher Scientific, Orlando, FL), and re-suspended in growth media at a density of 1.0 x 10^6 cells/ml. Experiments were initiated by back-culturing. Cells were suspended and centrifuged at 1000 rpm for 5 min. The cell pellet was re-suspended in the treatment medium at a concentration of 1.0 x 10^6 cells/ml, and plated

at 0.25 ml/well (low density plating) in 24 well Falcon plates (Fisher Scientific). Treatment media was growth media with either antisense or missense oligonucleotide (dissolved in artificial CSF) added in a concentration of 0.2 to 20 mM, preferrably 10 μM.

Equal amounts of total cell protein, from the missense and the antisense treatment groups, in Laemmli's buffer, were separated by SDS-PAGE (Simcha *et al* (1996) *J Cell Biol* **133**: 199-209). Protein was electro-transferred to PVDF membrane, and incubated with anti-mGluR$_1$ antibody (primary antibody) at 4 °C overnight. Primary antibody was washed off the membrane and incubated with peroxidase-conjugated secondary for 1 hour at room temperature. The antigen was visualized by chemiluminescence (Boehringer-Mannheim). The density of the bands was quantitatively determined using the Alpha Imager software.

These studies were performed using oligonucleotides of the present invention and the results are presented in Figures 19 – 23. These studies demonstrated that the cells treated with the antisense oligonucleotides produced less mGluR$_1$ in comparison to the cells treated with the missense control.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

TABLE 1: GROUPS FOR ANIMAL INFUSION

intrathecal infusion	dose (μg/day)	number of rats
rat antisense	5	8
	50	8
	500	8
	1000	8
rat missense	5	8
	50	8
	500	8
	1000	8
Human antisense	5	8
	50	8
	500	8
	1000	8
Human missense	5	8
	50	8
	500	8
	1000	8
Vehicle (artificial CSF)		8
non-infused controls		8

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WE CLAIM:

1. An antisense oligonucleotide derived from the sequence of a metabotropic glutamate receptor type 1 gene (mGluR1), wherein said oligonucleotide specifically binds to a portion of mRNA expressed from a gene encoding a mGluR1, or a splice variant thereof, and further wherein binding of said oligonucleotide to said mRNA is effective in decreasing the translation of said mRNA in a host cell expressing said gene.

2. An antisense oligonucleotide as in claim 1, wherein said sequence is any one of SEQ ID NO:1 to SEQ ID NO: 39.

3. The antisense oligonucleotide of claim 1, wherein said oligonucleotide has no more than 1 mismatch from the mRNA sequence to which it specifically binds.

4. The antisense oligonucleotide of claim 1, wherein at least one nucleotide phosphate of said oligonucleotide is substituted by a phosphorothioate, a methylphosphonate, or a C_{1-4} alkylphosphonate.

5. The antisense oligonucleotide of claim 1, wherein the 3' or 5' nucleotide of which further comprises a substituted acridine.

6. A compound comprising a salt or a hydrate of the antisense oligonucleotide of claim 1.

7. A composition comprising the antisense oligonucleotide of any one of claims 1 to 6.

8. The composition of claim 7 further comprising a pharmaceutical excipient.

9. A method for treating a patient having a disorder related to an elevated glutamate level, said method comprising administering to said patient an antisense oligonucleotide hybridizing to a mRNA encoding metabotropic glutamate receptor type 1 gene ($mGluR_1$), or a splice variant thereof.

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10. A method according to claim 9, wherein said antisense oligonucleotide is administered via an intrathecal, intravenous or subcutaneous route.

11. A method according to claim 10, wherein said $mGluR_1$ is from a species excluding rat.

12. A method according to any one of claims 9 to 11, wherein said $mGluR_1$ is human $mGluR_{1\alpha}$.

13. An oligonucleotide according to any one of claims 1 to 6, wherein said $mGluR_1$ is from a species excluding rat.

14. An oligonucleotide according to claim 13, wherein said $mGluR_1$ is human $mGluR_{1\alpha}$.

15. The antisense oligonucleotide of any one of claims 1 to 6 comprising a nucleotide sequence having from 13 to 22 bases in length, and hybridizing to a portion of said mRNA 3 bases prior to the initiation codon of said gene and continuing to the stop codon of said gene.

16. The use of an antisense oligonucleotide according to any one of claims 1 to 6, to treat chronic pain.

17. A use according to claim 16, wherein said pain is caused by injury or inflammation of a nerve.

18. A use according to claim 17, wherein said inflammation is caused by arthritis.

19. The use of an oligonucleotide according to claim 16 in combination with an opioid analgesic, to enhance effect of said opioid analgesic.

ABSTRACT

The present invention relates to an antisense oligonucleotides for metabotropic glutamate receptor type 1 ($mGluR_1$). There is provided oligonucleotides that hybridize to a mRNA encoding $mGluR_1$ or a splice variant thereof. There is also provided a method for treating a patient having a disorder related to an elevated glutamate level, such as chronic pain, or to enhance an opioid effect or to reduce opioid dependence and/or tolerance, which comprises the administration of such oligonucleotide. Specfic pain treatment is thereby obtained.

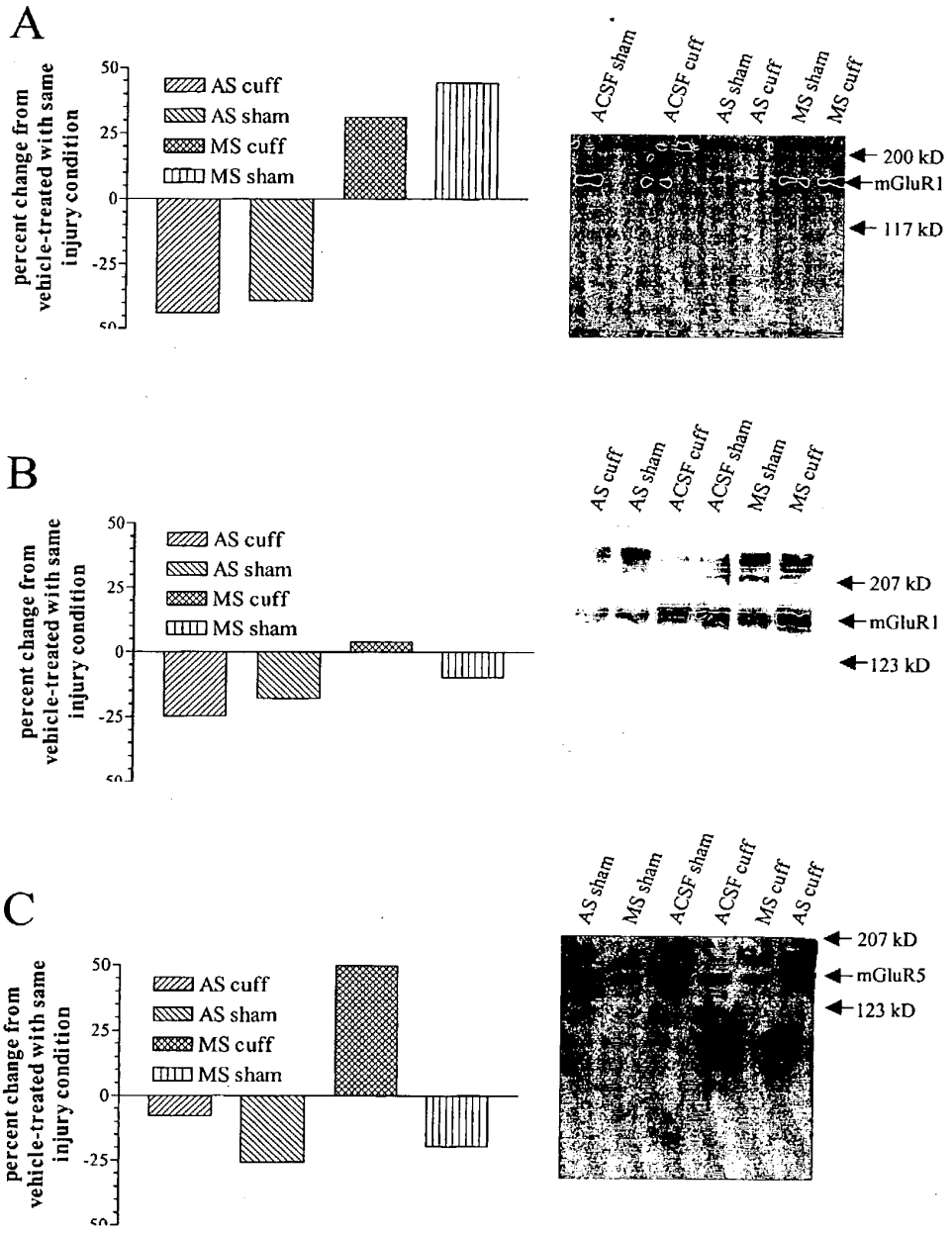
A
percent change from vehicle-treated with same injury condition
50
25
0
-25
AS cuff
AS sham
MS cuff
MS sham
ACSF sham
ACSF cuff
AS sham
AS cuff
MS sham
MS cuff
200 kD
mGluR1
117 kD
B
percent change from vehicle-treated with same injury condition
50
25
0
-25
AS cuff
AS sham
MS cuff
MS sham
AS cuff
AS sham
ACSF cuff
ACSF sham
MS sham
MS cuff
207 kD
mGluR1
123 kD
C
percent change from vehicle-treated with same injury condition
50
25
0
-25
AS cuff
AS sham
MS cuff
MS sham
AS sham
MS sham
ACSF sham
ACSF cuff
MS cuff
AS cuff
207 kD
mGluR5
123 kD

Figure 1

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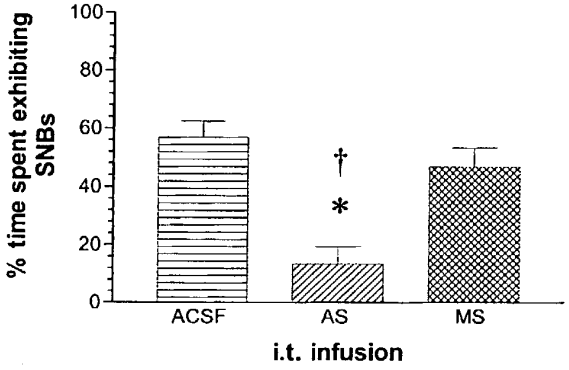
% time spent exhibiting SNBs
100
80
60
40
20
0
†
*
ACSF
AS
MS
i.t. infusion

Figure 2

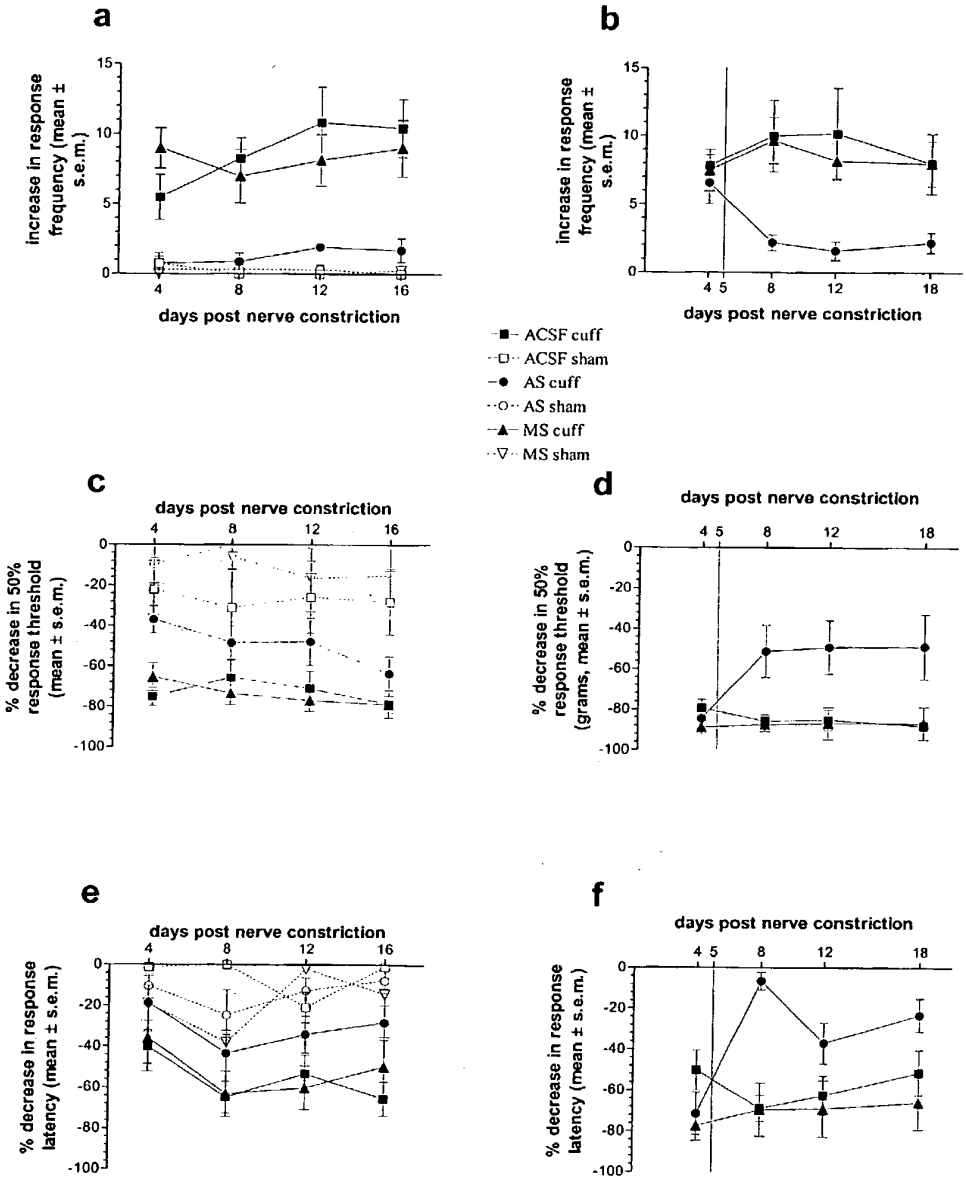
a
b
increase in response frequency (mean ± s.e.m.)
days post nerve constriction
ACSF cuff
ACSF sham
AS cuff
AS sham
MS cuff
MS sham
c
d
% decrease in 50% response threshold (mean ± s.e.m.)
% decrease in 50% response threshold (grams, mean ± s.e.m.)
e
f
% decrease in response latency (mean ± s.e.m.)

Figure 3

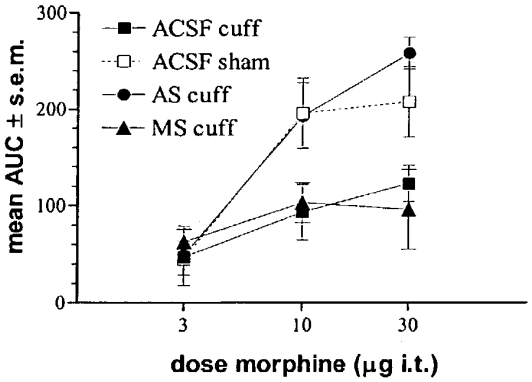
300
200
100
0
mean AUC ± s.e.m.
ACSF cuff
ACSF sham
AS cuff
MS cuff
3
10
30
dose morphine (µg i.t.)

Figure 4

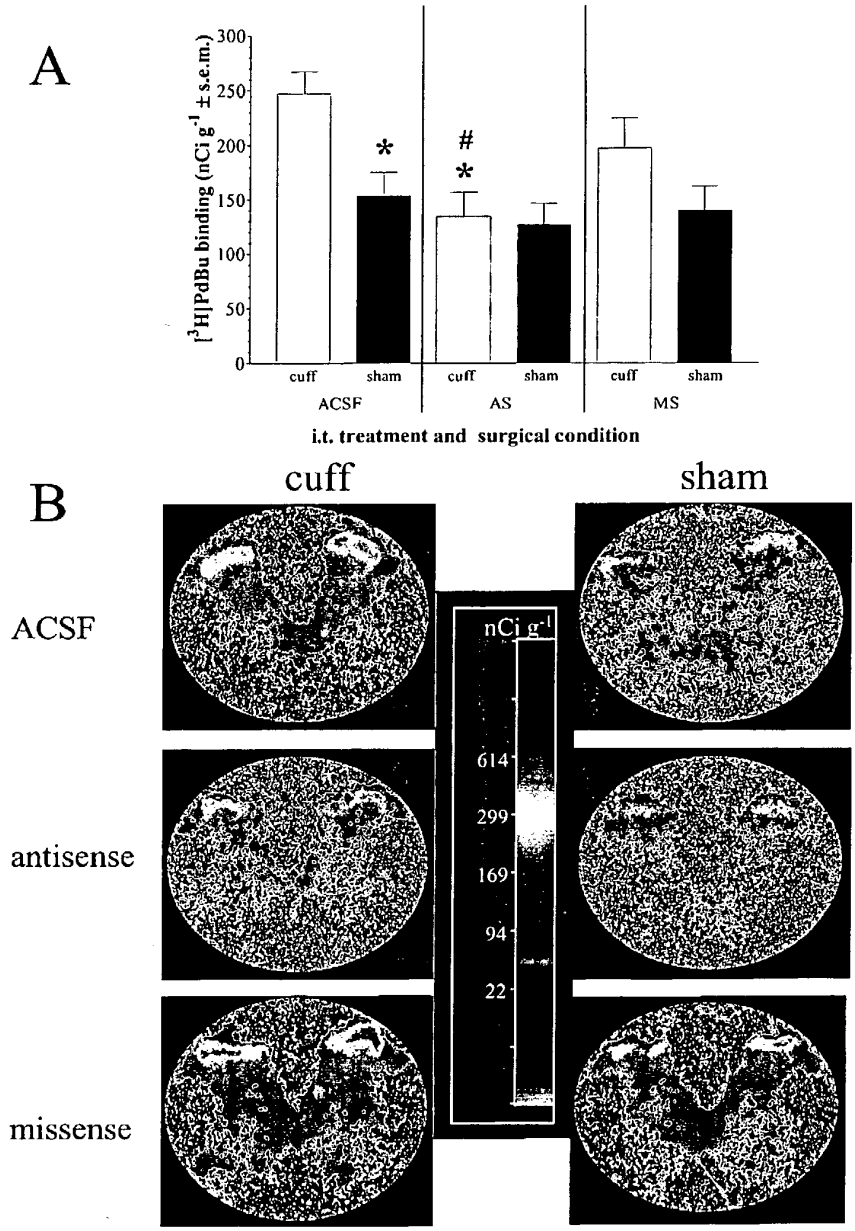
A
[3H]PdBu binding (nCi g-1 ± s.e.m.)
300
250
200
150
100
50
0
*
#
*
cuff
sham
cuff
sham
cuff
sham
ACSF
AS
MS
i.t. treatment and surgical condition
B
cuff
sham
ACSF
antisense
missense
nCi g-1
614
299
169
94
22

Figure 5

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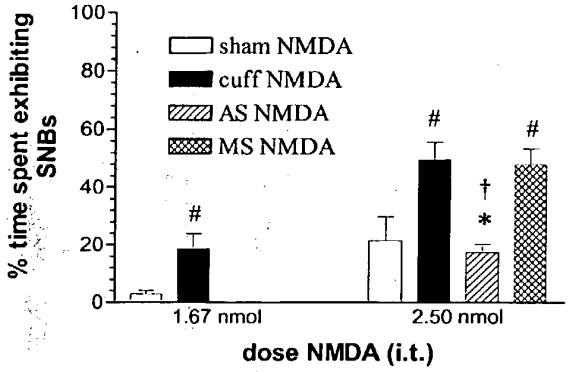

% time spent exhibiting SNBs
100
80
60
40
20
0
sham NMDA
cuff NMDA
AS NMDA
MS NMDA
#
#
#
†
*
1.67 nmol
2.50 nmol
dose NMDA (i.t.)

Figure 6

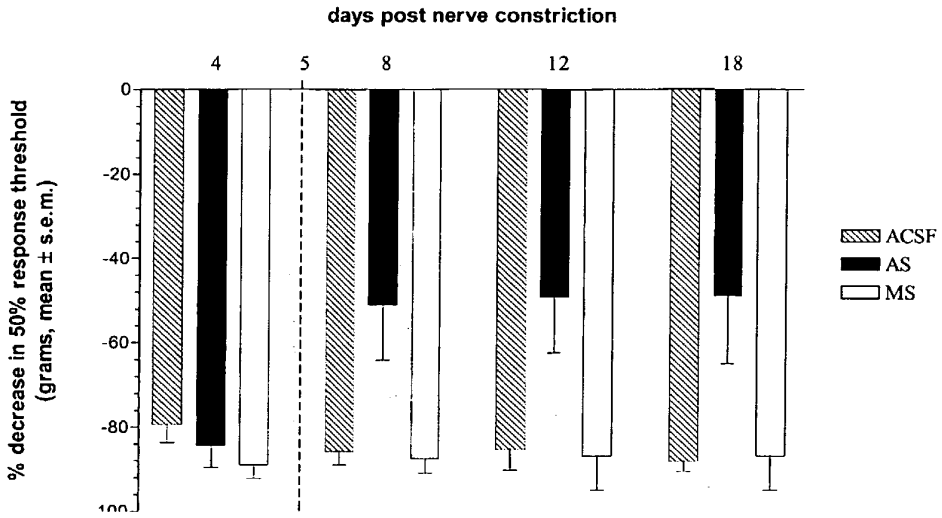

days post nerve constriction
4
5
8
12
18
0
-20
-40
-60
-80
% decrease in 50% response threshold
(grams, mean ± s.e.m.)
ACSF
AS
MS

Figure 7

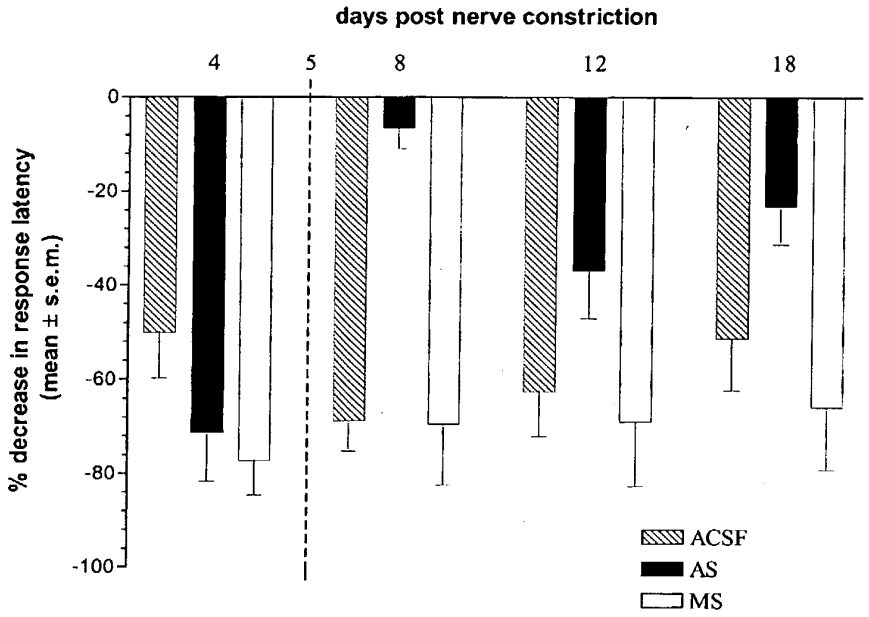

days post nerve constriction
4
5
8
12
18
0
-20
-40
-60
-80
-100
% decrease in response latency
(mean ± s.e.m.)
ACSF
AS
MS

Figure 8

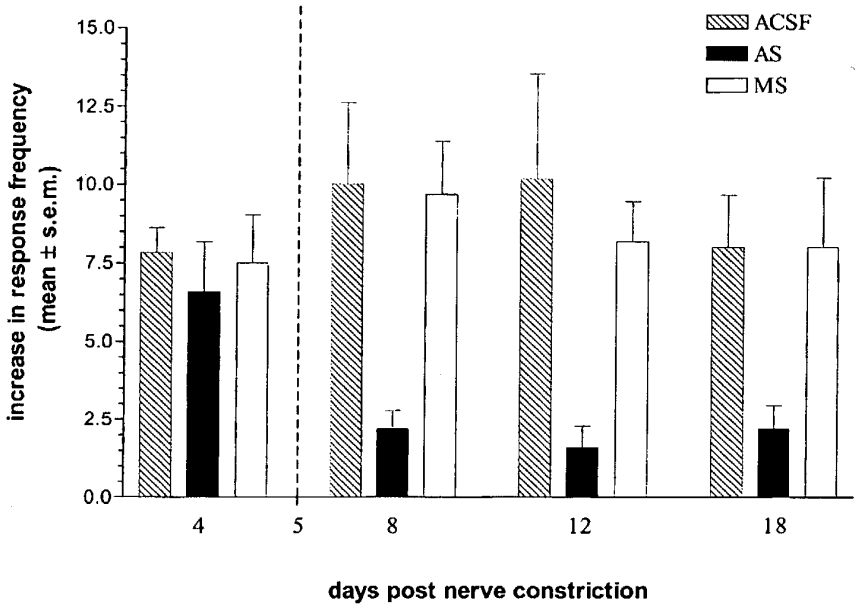

ACSF
AS
MS
15.0
12.5
10.0
7.5
5.0
2.5
0.0
increase in response frequency
(mean ± s.e.m.)
4
5
8
12
18
days post nerve constriction

Figure 9

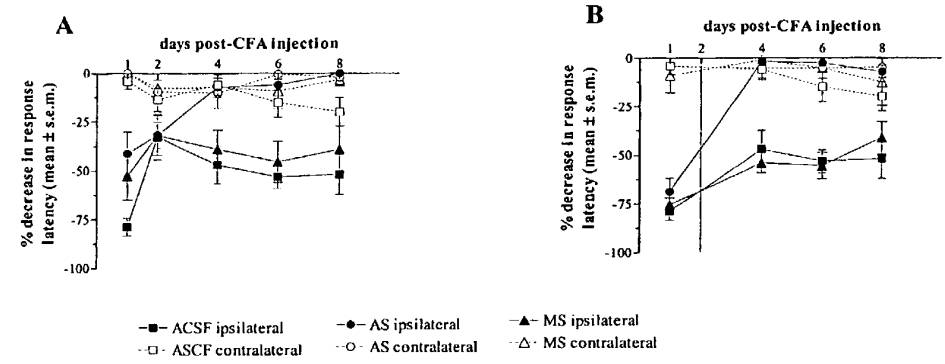

A
days post-CFA injection
% decrease in response latency (mean ± s.e.m.)
B
days post-CFA injection
% decrease in response latency (mean ± s.e.m.)
ACSF ipsilateral
ASCF contralateral
AS ipsilateral
AS contralateral
MS ipsilateral
MS contralateral
C
days post-CFA injection
% decrease in 50% response threshold (grams; mean ± s.e.m.)
D
days post-CFA injection
% decrease in 50% response threshold (grams; mean ± s.e.m.)

Figure 10

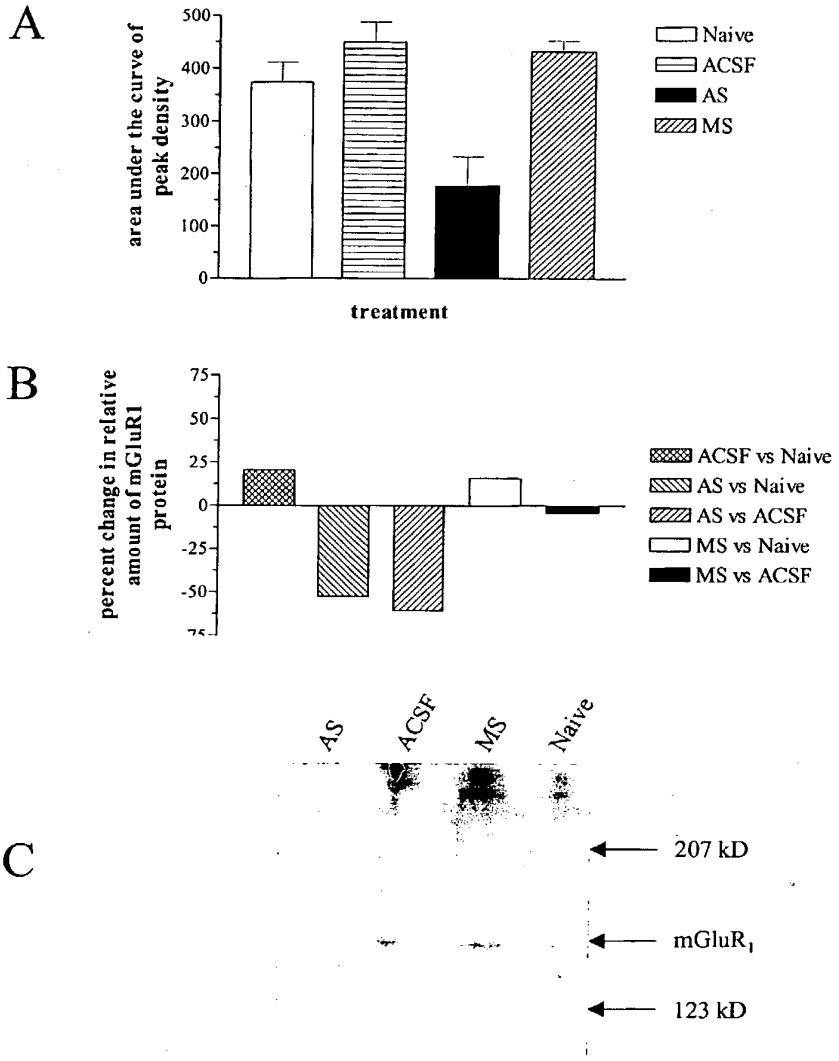

A
area under the curve of peak density
500
400
300
200
100
0
treatment
Naive
ACSF
AS
MS
B
percent change in relative amount of mGluR1 protein
75
50
25
0
-25
-50
ACSF vs Naive
AS vs Naive
AS vs ACSF
MS vs Naive
MS vs ACSF
C
AS
ACSF
MS
Naive
207 kD
mGluR1
123 kD

Figure 11

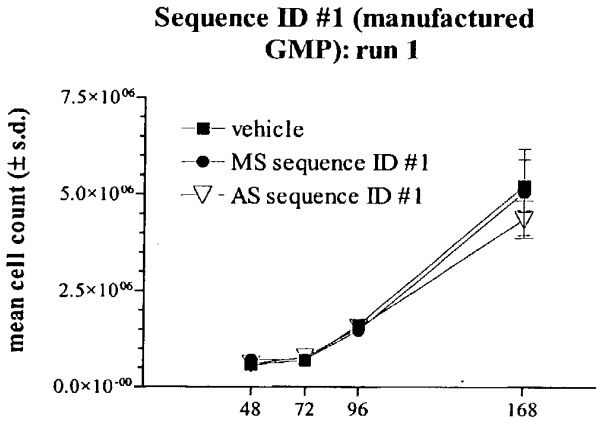

Sequence ID #1 (manufactured GMP): run 1
mean cell count (± s.d.)
7.5×10^06
5.0×10^06
2.5×10^06
0.0×10^-00
vehicle
MS sequence ID #1
AS sequence ID #1
48
72
96
168

Figure 12

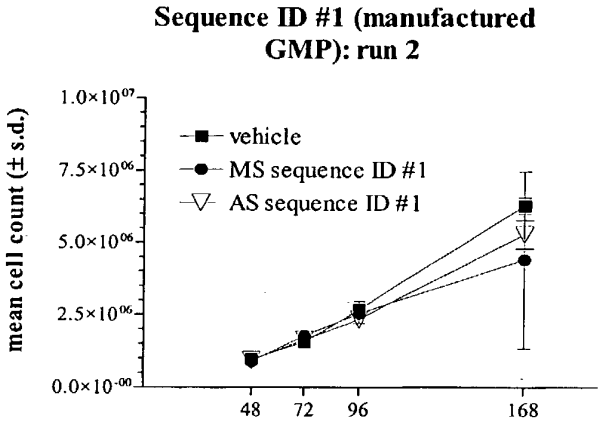

Sequence ID #1 (manufactured GMP): run 2
mean cell count (± s.d.)
1.0×10^07
7.5×10^06
5.0×10^06
2.5×10^06
0.0×10^-00
vehicle
MS sequence ID #1
AS sequence ID #1
48
72
96
168

Figure 13

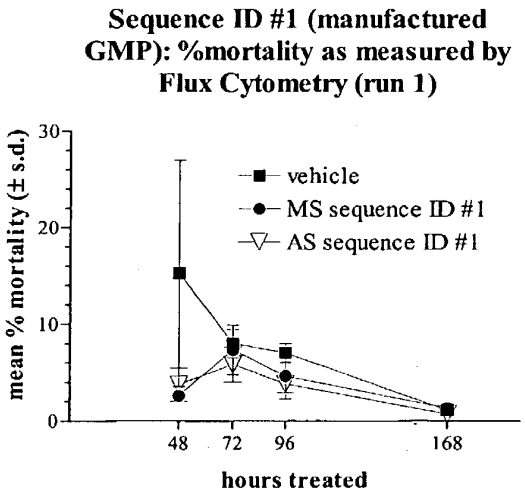

Sequence ID #1 (manufactured GMP): %mortality as measured by Flux Cytometry (run 1)
mean % mortality (± s.d.)
30
20
10
0
48
72
96
168
hours treated
vehicle
MS sequence ID #1
AS sequence ID #1

Figure 14

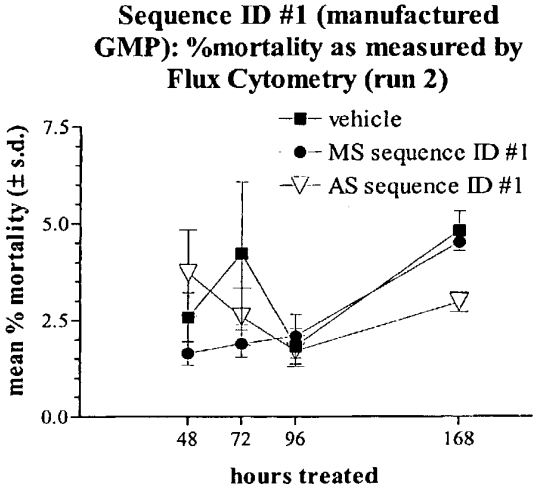

Sequence ID #1 (manufactured GMP): %mortality as measured by Flux Cytometry (run 2)
vehicle
MS sequence ID #1
AS sequence ID #1
mean % mortality (± s.d.)
7.5
5.0
2.5
0.0
48
72
96
168
hours treated

Figure 15

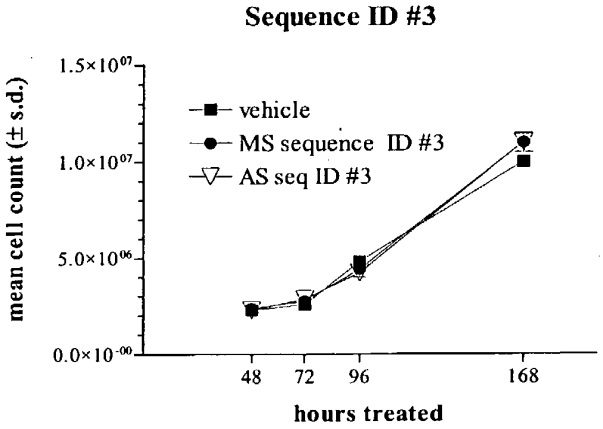

Sequence ID #3
1.5×10^07
1.0×10^07
5.0×10^06
0.0×10^-00
mean cell count (± s.d.)
vehicle
MS sequence ID #3
AS seq ID #3
48
72
96
168
hours treated

Figure 16

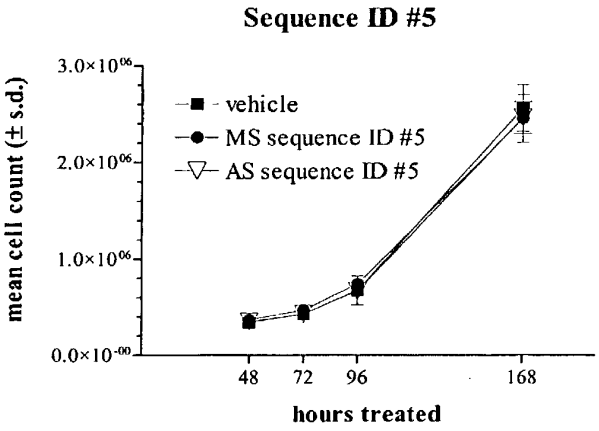

Sequence ID #5
3.0×10^06
2.0×10^06
1.0×10^06
0.0×10^-00
mean cell count (± s.d.)
vehicle
MS sequence ID #5
AS sequence ID #5
48
72
96
168
hours treated

Figure 17

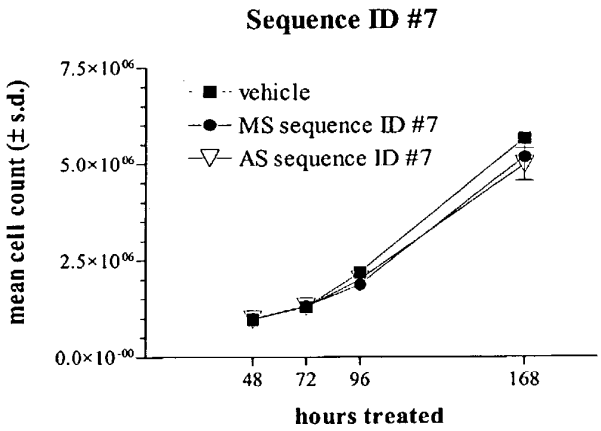
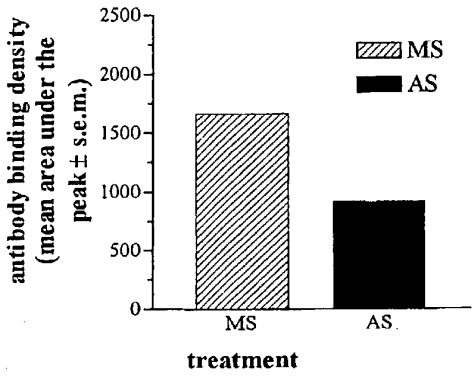

Sequence ID #7
7.5×10^06
5.0×10^06
2.5×10^06
0.0×10^-00
mean cell count (± s.d.)
vehicle
MS sequence ID #7
AS sequence ID #7
48
72
96
168
hours treated

Figure 18

Sequence ID #3



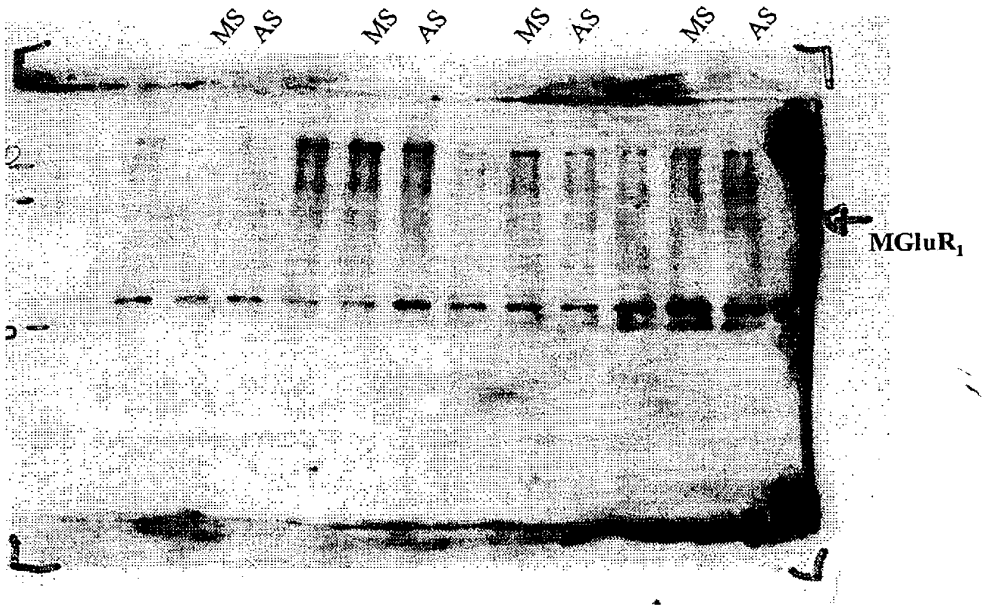
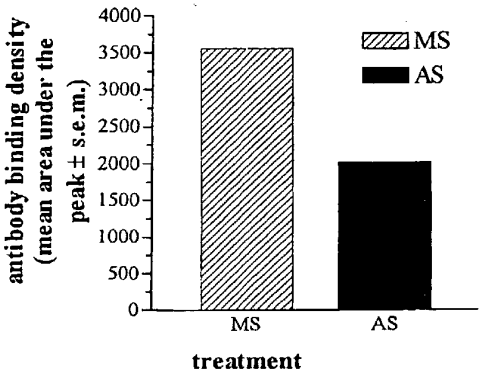


Figure 19

Sequence ID #5



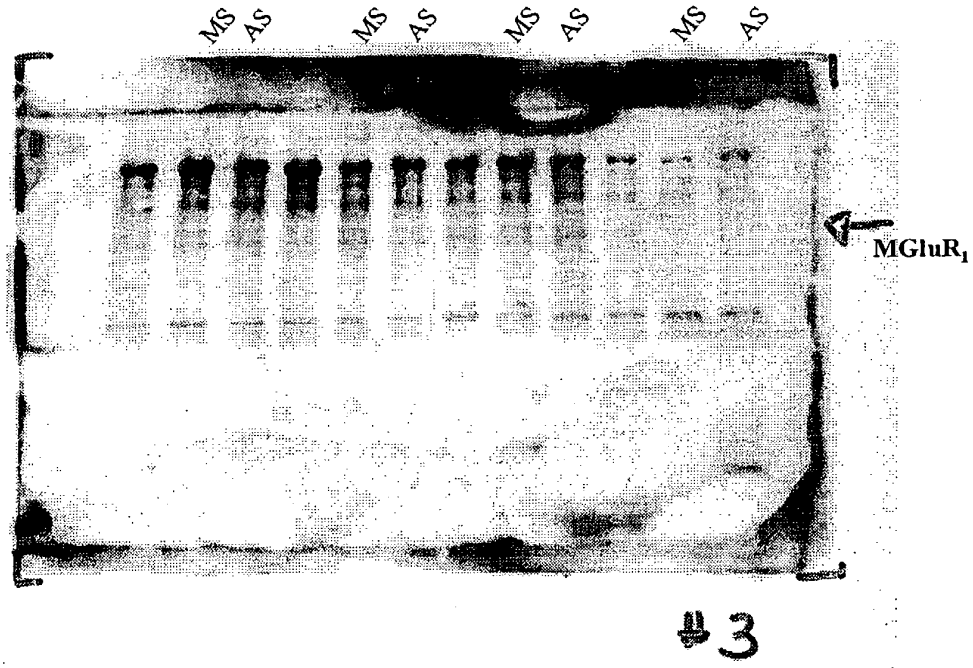
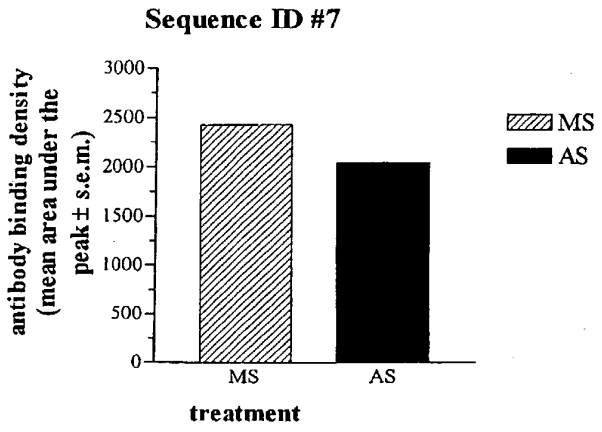


Figure 20


Sequence ID #7
antibody binding density (mean area under the peak ± s.e.m.)
3000
2500
2000
1500
1000
500
0
MS
AS
MS
AS
treatment

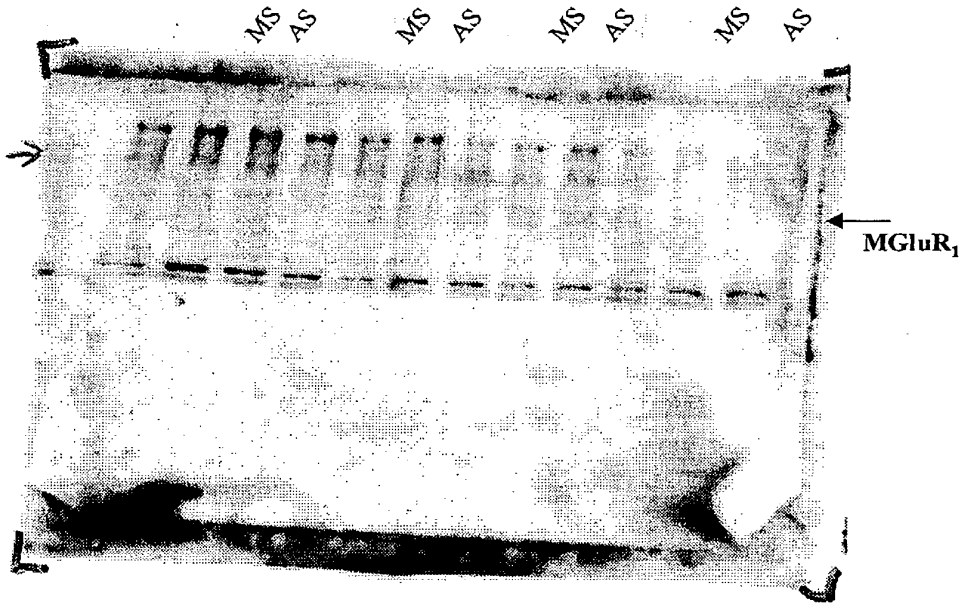
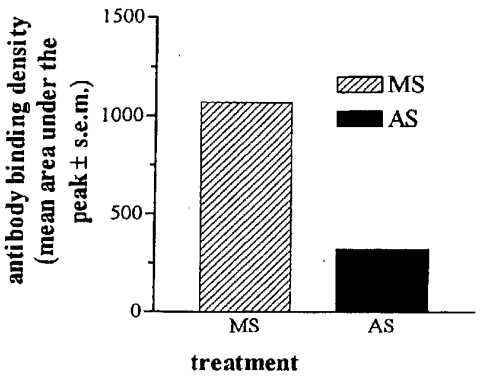

MS
AS
MS
AS
MS
AS
MS
AS
MGluR1

Figure 21

Sequence ID #1



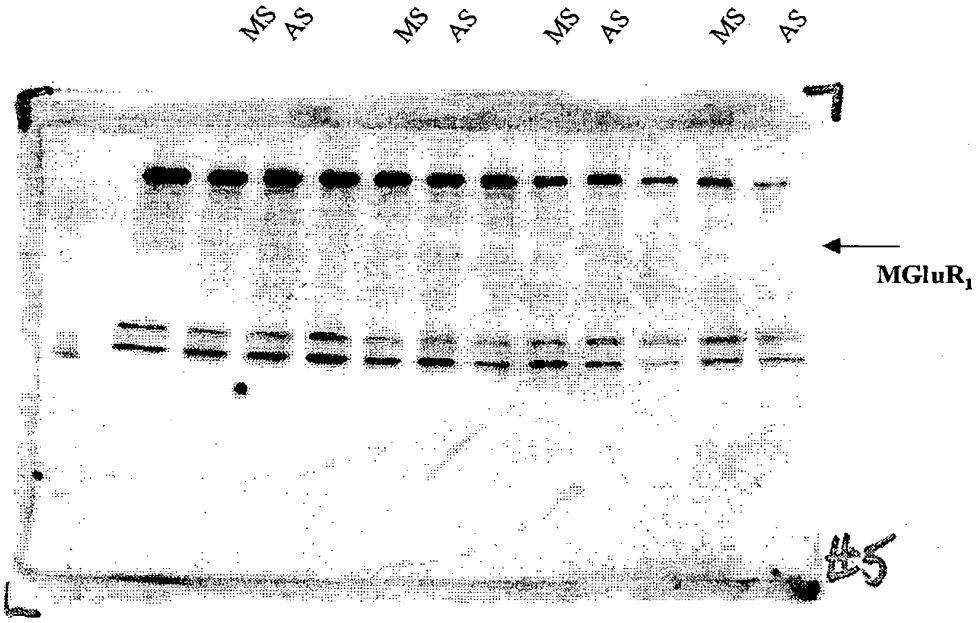
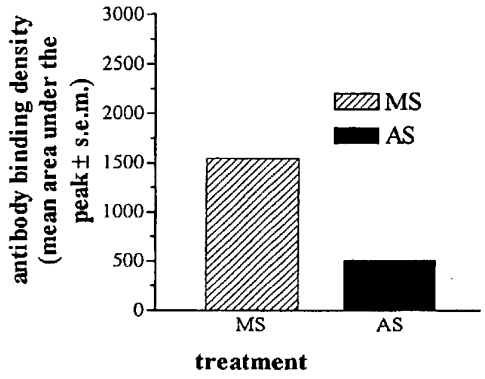


Figure 22

Sequence ID #1



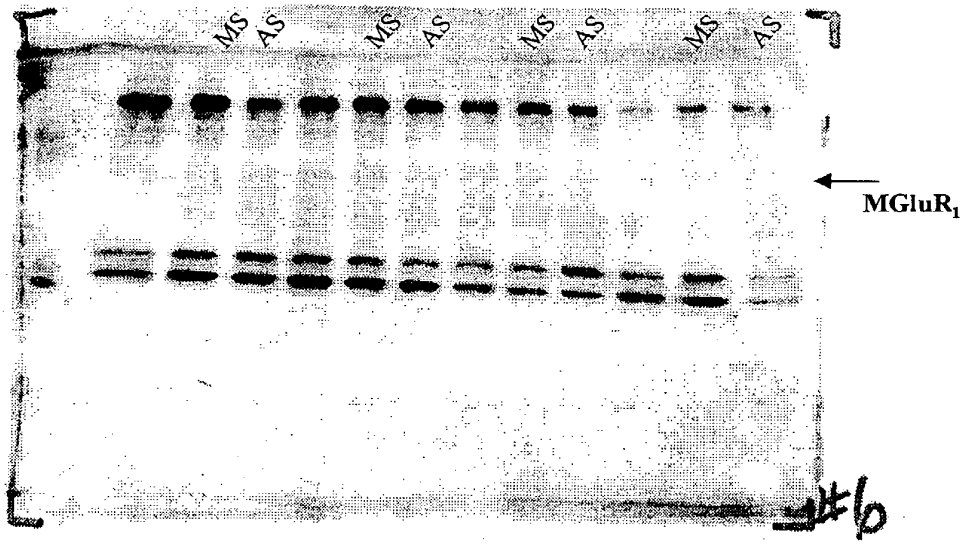


Figure 23

DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare:

That my residence, post office address and citizenship are as stated below next to my name.

That I verily believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural inventors are named below) of the subject matter which is claimed and for which a patent is sought on the invention entitled: **Antisense Oligonucleotides For Metabotropic Glutamate Receptor Type 1 (MGLUR1)** the specification of which (check one)

is attached hereto.

X was filed on _____ as Application Serial No. **10/031,308** and was amended on _____ (if applicable).

That I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

That I acknowledge the duty to disclose information known to be material to patentability of this application in accordance with Title 37, Code of Federal Regulations §1.56(a).

That I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate on this invention having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed ☐ Yes ☐ No
PCT/CA00/00824	PCT	17.07.200	
(Number)	(Country)	(Day/Month/Year Filed)	

That I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

United States Application(s)

_____	_____	_____
(Application Serial No.)	(Filing Date)	(Status)-(Patented, pending, abandoned)

That all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

I hereby appoint the following attorneys, with full power of substitution and revocation, to prosecute this application and to transact all business in the United States Patent and Trademark Office connected therewith and

application and to transact all business in the United States Patent and Trademark Office connected therewith and request that all correspondence and telephone calls in respect to this application be directed to: WELSH & KATZ, LTD., 120 South Riverside Plaza, 22nd Floor, Chicago, Illinois 60606-3913, Telephone No.: (312) 655-1500:

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Daniel R. Cherry	29,054
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Full name of sole or one joint inventor: TERENCE J. CODERRE

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Full name of sole or one joint inventor: S. ROBIN COHEN

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Inventor's signature: James L. Henry

Date: 02.09.06.

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N6G 4W5. GBN

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120 South Riverside Plaza
22nd Floor
Chicago, Illinois 60606-3913

5-00

Full name of sole or one joint investor: ANNELI VAINIO

Investor's signature: Anneli Vainio

Date: September 25, 2002

Residence and Post Office Address: 24 ... Rang Est, St-Anaclet, Quebec G0K 1H0 — Hiisintie 6 Nord, F 30190, [illegible], FRANCE

Citizenship: CANADA FINLAND

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10/031308
531 Rec'd PCT/PTO 15 JAN 2002

SEQUENCE LISTING

<110> Henry, James L
Fundytus, Marian E
Vaudreuil, Terrasse

<120> Oligonucleotide For Metabotropic Glutamate Receptor Type 1 (MGLUR1)

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<150> 60/144,004
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